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(54) Title: RECOMBINANT HUMAN METAPNEUMOVIRUS AND ITS USE

(57) Abstract: Recombinant HMPV (rHMPV) and related immunogenic compositions and methods are provided. The rHMPVs, including chimeric and HMPV vector viruses, provided according to the current disclosure are infectious and attenuated in permissive mammalian subjects, including humans. The rHMPVs are useful in immunogenic compositions for eliciting an immune response against HPIV, against one or more non-HMPV pathogens, or against a HMPV and a non-HMPV pathogen. Also provided are isolated polynucleotide molecules and vectors incorporating a recombinant HMPV genome or antigenome.



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RECOMBINANT HUMAN METAPNEUMOVIRUS AND ITS USE**PRIORITY CLAIM**

5 This application claims the benefit of U.S. Provisional Patent Application No. 60/451,119, filed February 28, 2003, and U.S. Provisional Patent Application No. 60/478,667, filed June 13, 2003. Both of the provisional applications are incorporated by reference herein in their entirety.

FIELD OF THE DISCLOSURE

10 This invention relates to the field of virology and, more specifically, to methods for producing recombinant human metapneumovirus (rHMPV), for producing replication competent derivatives with desirable properties such as attenuation and protective efficacy, and to uses of rHMPV in immunoprophylaxis and therapy.

15 **BACKGROUND**

Human metapneumovirus (HMPV) is a virus that was first recovered in the Netherlands from infants and children experiencing acute respiratory tract disease (van den Hoogen *et al.* Nat. Med. 7:719-724, 2001; De Jong *et al.* WO 02/057/302 A2).

20 HMPV is worldwide in prevalence and resembles human respiratory syncytial virus (RSV or HRSV) with regard to disease signs and the ability to infect and cause disease in the young infant as well as individuals of all ages (for a review, see Heikkinen and Jarvinen, Lancet 361:51-59, 2003).

HMPV is characterized as an enveloped virus with a genome that is a single negative strand of RNA of approximately 13 kb. The virus has been classified presumptively in the
25 Metapneumovirus genus, Pneumovirus subfamily, Paramyxovirus family of the Order Mononegavirales, comprising the nonsegmented negative strand RNA viruses or mononegaviruses. Mononegaviruses also are called nonsegmented negative strand RNA viruses. The Paramyxovirus family has two subfamilies, Paramyxovirinae and Pneumovirinae (also referred to as paramyxoviruses and pneumoviruses, respectively).

30 Several other mononegaviruses are important agents of respiratory tract disease in pediatric and other populations, for example, RSV and human parainfluenza virus types 1, 2 and 3 (HPIV1, HPIV2 and HPIV3). Although these viruses have some similarities with HMPV, their sequences are different from the HMPV viruses disclosed herein.

35 Since HMPV was described only recently, there is little documented experience with its propagation, manipulation and stability, and there are no well established or widely available reference strains, mutant strains, reported vaccine candidates, or reference virus-specific antibodies or comparable reagents or systems or experimental animals models to facilitate characterization. Because HMPV is associated with severe respiratory tract disease, there is a need to develop methods

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to engineer safe and effective vaccines to alleviate the serious health problems attributable to this pathogen, particularly among young infants.

BRIEF SUMMARY OF SPECIFIC EMBODIMENTS

5 HMPV is a significant agent of human respiratory tract disease. Methods and compositions are provided herein for recovering infectious, recombinant HMPV. Recombinant HMPV is disclosed herein, including the complete nucleic acid sequence encoding all of the protein products of HMPV. Compositions and methods for introducing defined, predetermined structural and phenotypic changes into an infectious HMPV are disclosed, as are attenuated forms of HMPV as well as forms of HMPV
10 that have been modified to have improved qualities relevant to immunogenicity, safety, protective efficacy, and breadth of vaccine coverage. Thus, methods are disclosed for generating an immune response against HMPV in a subject. The methods include administering to the subject an attenuated HMPV, thereby producing the immune response. Also described is the identification of a set of viral ORFs whose expression is sufficient to direct viral transcription and RNA replication, and to produce
15 infectious HMPV entirely from cDNA.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

20 BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a diagrammatic representation of the negative sense genomes of HMPV strain CAN97-83 (hereafter referred to as 83 or CAN-83) and RSV strain A2, showing a comparison of the two genomes. Individual proposed genes are shown as shaded boxes separated by proposed
25 intergenic regions represented by open bars. The 3' extragenic leader and 5' extragenic trailer regions also are shown as open boxes. Tentative HMPV gene assignments are based on ORFs whose potential protein exhibits partial amino acid sequence identity or structural similarity with a known protein from RSV. Note that HMPV lacks apparent counterparts to the NS1 and NS2 genes present in RSV (boxed), and that the putative F and M2 gene pair precedes the SH-G gene pair in HMPV but follows it in RSV (the difference in the position of SH-G is indicated with dotted lines). The M2 and
30 L genes overlap in RSV but may not do so in HMPV. In both viruses, the M2 gene contains two ORFs M2-1 and M2-2, depicted as filled bars. Amino acid lengths are shown for the deduced unmodified viral proteins. Each of the RSV proteins has been directly identified; none of the proposed HMPV proteins had been directly identified prior to the current disclosure. Abbreviations
35 (with HMPV assignments by analogy to RSV): N, major nucleocapsid protein; P, nucleocapsid phosphoprotein; M, inner virion matrix protein; M2-1 and M2-2, products of the first and second ORFs, respectively, in the M2 mRNA; SH, small hydrophobic protein; G, heavily glycosylated

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protein involved in attachment; F, fusion protein involved in penetration; L, large polymerase protein; NS1 and NS2, nonstructural protein 1 and 2, respectively. This and all subsequent diagrams are not to scale.

FIG. 2 is a diagram showing sequence alignments between the 3' leader (top) and 5' trailer (bottom) regions and flanking areas of the genome of HMPV strain 83 (SEQ ID NO: 18 and SEQ ID NO: 22, respectively), avian pneumovirus (APV or AMPV, SEQ ID NO: 19 and SEQ ID NO: 23, respectively), and HRSV strain A2 (SEQ ID NO: 20 and SEQ ID NO: 24, respectively). The sequences are in genome (negative) sense. Nucleotide assignments that are the same in two or more sequences between HMPV 83, APV and RSV are shaded; sequence gaps introduced to make optimal alignments are indicated by dashes. The bars over the left hand end of each sequence indicates regions whose spacing and sequence had not been previously determined for any HMPV. The partial sequences available for HMPV strain 00-1 3' leader and 5' trailer regions (SEQ ID NO: 17 and SEQ ID NO: 21, respectively) are indicated at the top: nucleotide assignments in strain 00-1 that are identical to those of HMPV 83 are indicated by asterisks while nucleotide differences are indicated. Sequences: APV (Randhawa *et al.*, J. Virol. 71:9849-54, 1997), RSV (Mink *et al.*, Virology 185:615-24, 1991), HMPV 00-1 (van den Hoogen *et al.*, Nat. Med. 7:719-24, 2001).

FIG. 3 is a diagram showing sequence alignments between the 3' and 5' ends of the genome of HMPV strain 83 (top; bases 1-41 of SEQ ID NO: 18 and bases 1-40 of SEQ ID NO: 22, respectively) and APV (bottom; bases 1-41 of SEQ ID NO: 19 and bases 1-42 of SEQ ID NO: 23, respectively), with complementary nucleotides boxed. This shows that the last 13 nucleotides of the two ends of APV are perfect complements, whereas HMPV unexpectedly has two noncomplementary nucleotides. Sequences for HMPV that were not previously available are indicated with solid lines above (leader, Le strand) or below (trailer, Tr strand) the sequence.

FIG. 4 is a diagram showing several examples of differences in nucleotide and amino acid lengths between HMPV strains 83 and 00-1. The differences in G are illustrated by partial amino acid sequences (upper alignment; amino acids 190-219 of SEQ ID NO: 6 and amino acids 190-236 of SEQ ID NO: 11, respectively) and partial nucleotide sequences (second alignment; bases 6871-6929 of SEQ ID NO: 1 and bases 6886-6964 of SEQ ID NO: 36, respectively). The sequences are numbered according to the amino acid sequence of HMPV 83 G (top alignment) or the complete nucleotide sequence of the antigenome of HMPV 83 (second alignment). Examples of differences include, (1) the SH protein for 83 is shorter by 4 aa due to an earlier stop codon; (2) the G protein for 83 is shorter by 17 aa due to an earlier stop codon, which is followed by a deletion in the downstream noncoding region; and (3) the intergenic region between the F and M2 genes of 83 is 13 nucleotides (nt) compared to 41 nt for 00-1. Taken together, these show that the G ORF and encoded G protein of strain 83 are shorter by the equivalent of 17 amino acids from that of strain 00-1.

FIG. 5 is a chart showing the percent amino acid sequence identity for the predicted, putative proteins of HMPV 83 compared to those of two other strains of HMPV (00-1, and 97-82),

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three different antigenic types of APV (A, B and C), the two subgroups of human RSV (A and B), bovine RSV (BRSV), and pneumonia virus of mice (PVM, a murine counterpart of HRSV). Note that HMPV is most closely related to APV C. ND indicates that a comparison was not done for the indicated subject.

5 **FIG. 6** is a taxonomy tree, illustrating putative phylogenetic relationships between HMPV and other paramyxoviruses based on comparisons of the amino acid sequences of proposed matrix M proteins. Pneumovirinae and Paramyxovirinae are the two subfamilies of the Paramyxovirus family. The other taxonomic names refer to genera. The numbers refer to the extent of amino acid sequence divergence (percentage non-identity) divided by 100; hence, 0.2 refers to 20% amino acid
10 differences. Note that a system for recovering and modifying infectious recombinant virus has not been reported for any member of genus Metapneumovirus even though the avian members of this genus have been known for almost 25 years.

FIG. 7 is a diagram illustrating the construction of a cDNA encoding the complete antigenome of HMPV strain 83, designed from the complete consensus genomic sequence. Three
15 separate subgenomic cloned fragments were created: fragment 1 contains the putative N, P and M genes and is bordered on the upstream (left) side by an added promoter for bacteriophage T7 RNA polymerase (T7p) and on the right hand side by an *NheI* site that was added to the putative M-F intergenic region as a marker. The sequence changes involved in introducing the *NheI* site are shown. The T7 promoter was designed to add three nonviral G residues to the 5' end of the antigenome, a
20 configuration chosen to improve the efficiency of the T7 promoter. Fragment 2 included the putative F, M2, SH and G genes and is bordered on the upstream side by the added *NheI* site and on the downstream side by a naturally occurring *Acc65I* site. Fragment 3 consists of the L gene followed by part of the hepatitis delta virus ribozyme (HDVribo) (Perrotta and Been, Nature 350:434-6, 1991) bordered by an *RsrII* site that occurs naturally within that ribozyme. The vector for cloning and
25 expressing the HMPV 83 antigenome cDNA was pBSKSII, which is a derivative of a modified Bluescript II KS + (Stratagene) vector described previously (Durbin *et al.*, Virology 71:4272-7, 1997) and contains the hepatitis delta virus ribozyme followed by a terminator for T7 RNA polymerase (T7t). This vector was modified by the insertion of a polylinker containing *AvaI*, *AatII*, *NheI*, and *Acc65I* sites, which in turn served to accept the cloned fragments 1, 2 and 3. The final cDNA encodes
30 the complete 13335-nucleotide HMPV 83 antigenome containing three added nonviral G residues at the 5' end. The complete pHMPV-83 recombinant plasmid ("antigenome plasmid") contains 16333 bp.

FIG. 8 is a diagram showing putative transcription signals for HMPV strain 83 based on the identification of semi-conserved sequence motifs located between the major ORFs in the complete
35 sequence. Sequences are in positive (mRNA) sense. The semi-conserved sequence motif that precedes most of the ORFs in the complete sequence is shown as the putative gene start (GS) signal, with some of the most highly conserved sequences shown in upper case letters and with flanking

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sequence on either side in lower case. The individual GS motifs are named according to the putative ORF that each precedes (SEQ ID NO: 1, bases 29-64 for N; bases 1237-1272 for P; bases 2154-2189 for M; bases 3041-3076 for F; bases 4698-4733 for M2; bases 5455-5490 for SH; bases 6206-6241 for G; and bases 7107-7142 for L). A consensus sequence is shown underneath (SEQ ID NO: 25).

5 For positions where alternative assignments can occur, these are listed below the consensus (SEQ ID NOs: 26 and 27). By this analysis, the major element of the GS signal is contained within sixteen nucleotides, of which positions 1, 3, 4, 6, 7, 9, and 14-16 were exactly conserved, with 14-16 also serving as potential translation start sites. Similarly, the semi-conserved sequence motif that follows most of the ORFs in the complete sequence is shown as the putative gene end (GE) signal, with some

10 of the most highly conserved sequences shown in upper case and flanking sequence in lower case. The individual GE motifs are named according to the ORF that each follows (SEQ ID NO: 1, bases 1230-1249 for N; bases 2141-2166 for P; bases 3002-3032 for M; bases 4680-4710 for F; bases 5440-5467 for M2; bases 6077-6107 for SH; bases 6912-6942 for G; and bases 13223-13253 for L). A consensus sequence is shown underneath (SEQ ID NO: 28). For positions where alternative

15 assignments can occur, these are listed below the consensus (SEQ ID NOs: 29 and 30). By this analysis, the major element of the GE signal is contained within 12-13 nucleotides, of which positions 1-3 and 10-12 are exactly conserved.

FIG. 9 is a diagram illustrating the construction of a transcription cassette containing the ORF for jellyfish GFP under the control of HMPV transcription GS and GE signals, and insertion of

20 this cassette into the HMPV strain 83 antigenomic cDNA clone following antigenomic position 41. Sequence representing part of the 3' leader region (bases 23-41 of SEQ ID NO: 1) and the putative N GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is shown at the bottom. The GFP transcription cassette consists of a 714-nucleotide cDNA containing the GFP ORF flanked on the upstream side by 16 nucleotides (which include the initiating ATG of the GFP ORF)

25 representing the conserved putative N GS signal (bases 42-57 of SEQ ID NO: 1) and flanked on the downstream side by 13 nucleotides representing the putative F GE signal (bases 4685-4697 of SEQ ID NO: 1; which include the termination codon of the GFP ORF) followed by the trinucleotide AGT designed to function as an intergenic (IG) region. The total length of the transcription cassette including the trinucleotide intergenic region is 748 nucleotides. The F GE signal contains a naturally

30 occurring *PacI* site (*italicized*).

FIG. 10 is a diagram illustrating the construction of expression vectors comprising support plasmids that contain ORFs corresponding to the proposed nucleocapsid and polymerase proteins of HMPV strain 83: N, P, M2-1 and L. These ORFs were clarified in part based on sequence relatedness to the respective RSV ORF (see, for example, Figure 5). Each ORF was placed under the control of a

35 T7 transcription promoter (T7p) and terminator (T7t). The L cDNA was assembled from two overlapping pieces. Each ORF conformed to the consensus sequence of HMPV 83.

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FIG. 11 is a diagram showing the strategy for recovery of a recombinant version of HMPV strain 83, in this case expressing GFP as an added marker gene. Plasmids encoding the HMPV-GFP antigenome and the N, P, L and M2-1 support plasmids are transfected into BSR T7/5 cells, which are a derivative of the baby hamster kidney-21 (BHK-21) cell line that was engineered to

5 constitutively express T7 RNA polymerase (Buchholz *et al.*, J. Virol. 73:251-259. 1999). The support plasmids were constructed to encode the proposed HMPV nucleocapsid and polymerase proteins. By this strategy, the expression of the antigenome together with HMPV nucleocapsid/polymerase proteins provides the components for the self-assembly of HMPV nucleocapsids functional for transcription and RNA replication. These nucleocapsids then express all of the HMPV RNAs and

10 proteins, leading to a productive infection that produces rHMPV.

FIG. 12A is a digitized image of an agarose gel showing that the recovered recombinant HMPV-GFP (strain 83) contains the *NheI* site that was engineered into the antigenome cDNA and is not present in biologically-derived strain 83 parent. LLC-MK2 cells in T25 flask were infected with an aliquot of the supernatant from the transfected BSR T7/5 cells (first passage) and incubated at

15 32°C in the presence of 5 µg/ml trypsin. Eleven days post-infection, 1 ml of the supernatant was harvested, clarified by centrifugation at 1500 rpm for 5 minutes and used to extract the viral genomic RNA with the QIAamp viral RNA purification kit (QIAGEN) according to the manufacturer's recommendations. The RNA was subjected to reverse transcription (RT) using a positive sense primer designed to hybridize within the M gene (nucleotides 2719 to 2738 in the HMPV antigenome exclusive of GFP). Polymerase chain reaction (PCR) was performed using the RT primer and a

20 reverse primer designed to hybridize within the F gene (nucleotides 3894 to 3876 in the HMPV antigenome exclusive of GFP). An aliquot of each RT-PCR reaction was subjected to digestion with *NheI*. The RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Lanes: 1 and 7, molecular weight marker; 2, a negative control in which RNA from the

25 recombinant HMPV-GFP-infected cell supernatant was assayed with the omission of RT enzyme; lanes 3 and 4, RT-PCR products from RNA representing recombinant HMPV-GFP and biologically-derived HMPV, respectively; lanes 5 and 6, RT-PCR products from RNA representing recombinant HMPV-GFP and biologically-derived HMPV, respectively, that had been subjected to digestion with *NheI*.

FIG. 12B is a digitized image of a nylon membrane illustrating Northern blot analysis of GFP mRNA expressed by the rHMPV-GFP virus. LLC-MK2 cells were mock-infected (lanes 1 and 5) or infected at an MOI of 3 PFU per cell with biologically-derived HMPV83 (lanes 2 and 6), rHMPV (lanes 3 and 7), or rHMPV-GFP (lanes 4 and 8). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon and

35 analyzed by hybridization to double-stranded ³²P-labeled DNA probe specific to the GFP (lanes 1-4) or M (lanes 5-8) gene. The identities and calculated sizes of individual RNA species are indicated.

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This showed that the GFP transcription cassette was expressed predominantly as a single mRNA of the appropriate size to be a monocistronic GFP mRNA.

FIG. 13 is a graph illustrating multi-step growth of the recovered recombinant HMPV (strain 83) (rHMPV) compared with biologically-derived HMPV 83. LLC-MK2 cells were inoculated with 0.01 plaque forming units (pfu) per cell and incubated at 32°C in the presence of 5 µg/ml trypsin. Aliquots were taken at 24 h intervals, flash-frozen, and viral titers were measured by plaque assay and immunostaining with convalescent serum from HMPV-infected hamsters. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown.

FIG. 14 is a graph illustrating multi-step growth of recombinant HMPV-GFP compared to recombinant HMPV in LLC-MK2 cells, performed as described above for Figure 13. Two separate infections (a and b) were monitored for each virus, and each aliquot was titrated in duplicate and the mean is shown.

FIG. 15 is a diagram illustrating deletion of the SH and G genes singly and in combination from rHMPV and rHMPV-GFP (strain 83), each containing introduced *BsiWI* and *BsrGI* restriction sites. The region of the antigenomic cDNA clone containing the putative SH and G ORFs is illustrated, together with proposed GS and GE transcription signals and proposed intergenic regions. *BsiWI* and *BsrGI* sites were introduced into the putative M2-SH and SH-G, respectively, intergenic regions of HMPV-GFP. In each case, the mutant sequence (nucleotide substitutions in small case) is shown below the wild type sequence. The restriction sites are numbered according to the position of the first residue in the wild type HMPV sequence (SEQ ID NO: 1) exclusive of GFP, a convention that will be followed throughout this disclosure. The naturally-occurring *Acc65I* sequence also is shown. Note that cleavage of each of these restriction sites by its cognate enzyme leaves a compatible overhang, namely GTAC, that is compatible with each of the others. Thus, the genes can be readily deleted by cutting with the appropriate pair of enzymes and religating. This results in small changes in the length of the relevant intergenic region, as noted. Each of these antigenomic cDNAs was successfully used to recover infectious mutant virus.

FIG. 16 is a digitized image of an agarose gel showing that the recovered recombinant HMPV-GFP ΔSH, ΔG and ΔSH/G viruses each contain a deletion of the appropriate size in the region of the SH and G genes. Following the procedure described above for Figure 12, viral RNA was subjected to RT-PCR using primers that span the SH and G genes; specifically a positive sense primer designed to hybridize within the M2 and a negative sense primer designed to hybridize within the L gene. The RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Lanes: 1 and 8, molecular weight markers; lane 3, a negative control in which RNA from recovered recombinant HMPV was subjected to the RT-PCR procedure with the omission of RT enzyme; lanes 2 and 4-7, RT-PCR products representing the indicated viruses.

FIG. 17A is a graph illustrating multi-step growth of rHMPV-GFP-ΔSH, rHMPV-GFP-ΔG, and rHMPV-GFP-ΔSH/G compared to rHMPV and rHMPV-GFP, performed in LLC-MK2 cells as

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described above for Figure 13. Since the deletions had been introduced into rHMPV-GFP, rHMPV-GFP is the "wild type" equivalent for comparison.

FIG. 17B is a graph illustrating the efficiency of replication of biologically-derived HMPV83 and recombinantly-derived rHMPV, rHMPV Δ SH, rHMPV Δ G, and rHMPV Δ SH/G in the upper (nasal turbinates) and lower (lungs) respiratory tract of Golden Syrian hamsters. Animals in groups of 12 were infected intranasally with the following viruses at a dose of 5.0×10^5 TCID₅₀ per animal in 0.1 ml: biologically derived HMPV83, rHMPV, rHMPV Δ SH, and rHMPV Δ SH/G, and the following virus at 1.6×10^5 TCID₅₀ per animal in 0.1 ml: rHMPV Δ G. Six animals from each group were sacrificed on days 3 and 5 and the nasal turbinates and lungs were recovered and analyzed by limiting dilution to determine the viral titer. This showed that the rHMPV replicated in vivo with an efficiency similar to that of its biologically-derived parent (HMPV83), that the replication of the rHMPV Δ SH virus was not significantly reduced, and that the replication of the rHMPV Δ G and Δ SH/G viruses were strongly reduced, although virus replication was detectable in each case.

FIG. 17C is a graph illustrating the protective efficacy of immunization with the indicated HMPV deletion mutant viruses. Golden Syrian hamsters in groups of 6 were infected as described in Figure 17B and, 27 days later, serum samples were taken and analyzed to determine the titers of HMPV-neutralizing serum antibodies. These titers are shown at the bottom. The animals were then challenged on day 28 by the intranasal instillation of $5.0 \log_{10}$ TCID₅₀ per animal of biologically-derived wild type HMPV83. Three days later, the animals were sacrificed and the nasal turbinates and lungs were harvested, homogenized, and analyzed by limiting dilution to determine virus titers. This showed that there was no detectable replication of the challenge virus in animals that has received rHMPV or rHMPV Δ SH, whereas animals that initially had received rHMPV Δ G or rHMPV Δ SH/G had no detectable challenge virus replication in the lungs and had reduced replication in the nasal turbinates compared to the control that had not been previously infected.

FIG. 18A is a diagram illustrating ablation of the putative M2-2 ORF in infectious rHMPV and rHMPV-GFP (strain 83), each containing an introduced *Bsi*WI site. The region of the antigenomic cDNA clone containing the putative M2-1 and M2-2 ORF is illustrated, with each ORF depicted by a solid horizontal line. Two nucleotide substitutions were made at positions 5236 and 5248 (SEQ ID NO: 1; numbered according to the complete antigenomic cDNA exclusive of GFP) that ablate two potential translation initiation codons for the M2-2 ORF. A third substitution was made at position 5272 (SEQ ID NO: 1) that introduces an in-frame stop codon. In addition, nucleotides 5289-5440 (SEQ ID NO: 1) were deleted, removing most of the M2-2 ORF. The Δ M2-2 mutant was successfully recovered in both the rHMPV and rHMPV-GFP backbones and designated rHMPV Δ M2-2 and rHMPV-GFP Δ M2-2, respectively.

FIG. 18B is a graph illustrating evaluation of multi-step growth kinetics of the recovered viruses *in vitro*. The rHMPV Δ M2-2 and rHMPV-GFP Δ M2-2 viruses were compared with rHMPV and rHMPV-GFP with regard to multi-step growth kinetics and yield in LLC-MK2 cells as described

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above for Figure 13. rHMPV Δ M2 clones #1 and #2 represent virus derived independently from two sister cDNA clones.

FIG. 18C is a digitized image of a nylon membrane illustrating Northern blot analysis of intracellular RNAs expressed by rHMPV-GFP and rHMPV-GFP Δ M2-2. Replicate monolayers of LLC-MK2 cells were infected at an MOI of 3 PFU per cell with either virus as indicated and incubated at 32°C. Monolayers were harvested at 9, 24, 36, 48, and 72 h post infection as indicated and processed for purification of intracellular RNA. The RNA preparations were analyzed by Northern blot hybridization with strand-specific riboprobes representing the N gene: the upper panels detect hybridization with the negative-sense probe and thus represent antigenome and N-related mRNA, as indicated, and the bottom panels detect hybridization of a replicate set of gel lanes (that had been transferred in parallel onto the same membrane, which was then cut and the replicate sets hybridized separately) with positive-sense riboprobe and represent genome. Beneath the Northern blot are calculations from four experiments involving hybridization with a probe specific for N or for F, as indicated. In each experiment, the amount of total N-related or F-related mRNA for each time point was divided by the amount of antigenome from a replicate gel lane (that had been transferred in parallel onto the same membrane, which was then cut and the replicas hybridized separately). Then, each time point for the rHMPV-GFP Δ M2-2 virus was normalized relative to the corresponding time point for rHMPV-GFP as 1.0. Thus, this provides a comparison of the efficiency of mRNA expression for the Δ M2-2 mutant compared to wild type HMPV.

FIG. 19 is a diagram illustrating the introduction of single amino acid substitutions into the Cys3-His1 motif of the M2-1 protein of rHMPV-GFP (strain 83). This motif consists of three cysteine residues (C7, C15 and C21) and one histidine residue (H25); it is also found in RSV and other pneumoviruses, and thus represents a conserved pneumovirus motif. The amino acid sequence shown is from methionine (M) 1 to asparagine (N) 26 of the deduced complete M2-1 amino acid sequence (SEQ ID NO: 4). Each of these mutants was successfully recovered as infectious recombinant virus.

FIG. 20 is a graph illustrating multi-step growth of derivatives of rHMPV-GFP containing mutations in the Cys3-His1 motif of the M2-1 ORF, compared to rHMPV-GFP and rHMPV, performed in LLC-MK2 cells as described above for Figure 13. The mutants involve single amino acid point mutations in the Cys3-His1 motif: C7S, Y9S, C15S, N16S and H25S. The mutations were introduced into rHMPV-GFP, and thus rHMPV-GFP is the "wild type" or parental equivalent for comparison.

FIG. 21 is a diagram illustrating silencing of the M2-1 ORF in rHMPV-GFP (strain 83) by replacing the ATG translational start site (shaded) with a TAG translational termination codon, and by replacing additional ATG triplets (underlined) in each reading frame with stop codons. The top line shows the nucleotide sequence of the upstream end of the M2-1 mRNA (corresponding to nucleotides 4711 to 4775 in the complete HMPV antigenome sequence; SEQ ID NO: 1). The second

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line shows nucleotide substitutions (lower case letters) introduced into the M2-1 sequence of rHMPV-GFP to yield rHMPV-GFP-ΔM2-1. The third line shows the first 17 amino acids of the HMPV M2-1 protein (SEQ ID NO: 4), and the fourth line shows coding changes introduced by the point mutations in rHMPV-GFP-ΔM2-1, with termination codons in the M2-1 ORF indicated by asterisks. Note that the next in-frame ATG in the M2-1 ORF is at codon 134 out of the total of 187 codons in the ORF (SEQ ID NO: 4). This mutant was successfully recovered as infectious recombinant virus.

FIG. 22A is a diagram illustrating deletion of the complete M2 gene in infectious rHMPV-GFP (strain 83) containing an introduced *Bsi*WI site. The region of the antigenomic cDNA clone containing the putative M2-1 and M2-2 ORF is illustrated, with each ORF depicted by a solid horizontal line. Nucleotides 4701 to 5459 (SEQ ID NO: 1), representing a total of 759 nucleotides, were deleted, resulting in the mutant rHMPV-GFP-ΔM2(1+2). This mutant was successfully recovered as infectious recombinant virus.

FIG. 22B is a graph illustrating a comparison of the efficiency of growth of viruses with alterations in the coding of M2-1 and/or M2-2 proteins in LLC-MK2 cells (upper panel), which are competent for expressing type I interferons versus Vero cells (lower panel), which lack the interferon structural genes. Cells were infected with 0.01 PFU per cell and incubated at 32°C in the presence of 5 μg/ml trypsin. Aliquots were taken at 24 h intervals, flash-frozen, and viral titers were measured by plaque assay and immunostaining with convalescent serum from hamsters that had been infected with HMPV.

FIG. 22C is a chart demonstrating increased sensitivity of HMPV ΔM2(1+2) and ΔM2-2 mutants to type I interferon. Replicate cultures of Vero cells were treated overnight with the indicated amount of interferon per 1.5×10^6 cells. The cells were infected with the indicated amount of wild type rHMPV-GFP, rHMPV-GFPΔM2(1+2), rHMPV-GFPΔM2-2, or rgRSV, the last being a recombinant RSV that expresses the GFP gene. The GFP marker was used to visually monitor virus growth during the experiment to judge the appropriate time of harvest, and is otherwise irrelevant to this experiment. The cells were harvested on day 4 and the yield of each virus was determined and compared with additional replicate cultures that had been mock-interferon treated, infected and harvested in parallel. The results are expressed as the fold reduction of each interferon-treated culture compared to its untreated counterpart.

FIG. 23A is a diagram illustrating the construction of two rearrangements of the genes of rHMPV-GFP (strain 83). The wild type gene order is shown on top for the M-L region of the rHMPV-GFP antigenomic cDNA clone. The restriction sites shown are the ones introduced as illustrated above in Figures 7 and 15 (the restriction sites are numbered according to the position of the first residue in the wild type HMPV sequence, SEQ ID NO: 1, exclusive of GFP), and were used to rearrange the order of the putative SH and G gene pair. Religations involving *Nhe*I were not compatible with the other sites and necessitated fill-in of each end followed by blunt end ligation.

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Two examples of gene rearrangements are shown: in the mutant called "Order 1", the positions of the SH-G gene pair and the F-M2 gene pair were swapped, resulting in a local gene order that mimics that of RSV, namely M-SH-G-F-M2-L. This mutant was successfully recovered as infectious virus. In a subsequent mutant, "Order 2", the same RSV-like gene order was achieved, but the SH-G gene pair was duplicated.

FIG. 23B is a graph illustrating the multi-step growth kinetics of one of these mutants, rHMPV-GFP-Order #1 compared to its direct parent rHMPV-GFP, as well as rHMPV, performed as described above for Figure 13. Clones #3 and #13 represent viruses derived independently from two sister cDNA clones.

FIG. 24A is a diagram illustrating shifting of the F and/or G genes of HMPV (strain 83) from their natural positions as the fourth and seventh genes, respectively, to promoter-proximal positions 1 or 2. As illustrated in the upper box, the putative F and G ORFs were engineered to be flanked by putative GS (bases 3054-3069 and 6219-6234 of SEQ ID NO: 1, respectively) and GE (bases 4685-4697 of SEQ ID NO: 1) signals and inserted individually into the HMPV antigenomic cDNA clone. Sequence representing part of the 3' leader region (bases 23-41 of SEQ ID NO: 1) and the putative N GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is shown. Note that the choice of transcription signals and insertion site was the same as for the rHMPV-GFP construct shown above in Figure 9, and places the inserted gene as the first in the gene order. As illustrated in the lower box, the putative F and G ORFs were inserted as a pair, in the order G1-F2 or its converse F1-G2, using the same transcription signals and insertion site as in the single-gene rearrangements. Infectious virus was successfully recovered from each of the constructs. These viruses were designated rHMPV-F1, -G1, -F1G2, and G1F2, with the number indicating the position of the gene in the gene order. In the virus designation, the "-" symbol preceding the shifted gene indicates that the shifted gene was removed from its normal position in the gene order.

FIG. 24B is a graph illustrating multi-cycle growth kinetics of the recovered viruses *in vitro*. The recovered rHMPV-G1, F1, F1G2, and G1F2 viruses were compared to their rHMPV parent with regard to multi-step growth in LLC-MK2 cells as described above for Figure 13.

FIG. 25A is a diagram showing representations of rHMPV in which one or more extra copies of the F and/or G gene was placed in promoter proximal positions 1, 2 or 3 in addition to the F and/or G gene present in the normal genome position. The G or F genes were inserted individually into promoter proximal position 1 (rHMPV+G1 or +F1), or were inserted as a pair into positions 1 and 2 in each of the two possible orders (rHMPV+G1F2 or +F1G2), or were inserted as the pair G1F2 with an additional copy of F in the third position (rHMPV+G1F2,3). The detailed structure of the gene insertions were as shown in Figure 24A. In the designations, the "+" symbol before the named gene or genes indicates that it/they were additional to the one or ones in the normal position. The added genes are shaded.

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FIG. 25B is a graph illustrating multi-cycle growth kinetics *in vitro*. The recovered viruses were compared to their rHMPV parent and to rHMPV-GFP with regard to multi-step growth in LLC-MK2 cells as described above for Figure 13.

FIG. 25C is a digitized image of a nylon membrane illustrating Northern blot analysis of intracellular mRNAs expressed by rHMPV+G1F23. LLC-MK2 cells were mock-infected (lanes 1, 5 and 9) or infected at an MOI of 3 PFU per cell with HMPV83 (lanes 2, 6 and 10), rHMPV (lanes 3, 7 and 11), or rHMPV+G1F23 (lanes 4, 8 and 12). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon and analyzed by hybridization to double-stranded ³²P-labeled DNA probe specific to the F (lanes 1-4), G (lanes 5-8), or M (lanes 9-12) gene. The identities and calculated sizes of individual RNA species are indicated.

FIGS. 26A-26F are diagrams illustrating amino acid locations in the strain 83 HMPV L protein (SEQ ID NO: 7) that are targets for mutagenesis based on mapping of attenuating mutations in a heterologous virus (HRSVA2, SEQ ID NO: 13; HPIV3, SEQ ID NO: 14; HPIV1, SEQ ID NO: 15; or BPIV3, SEQ ID NO: 16, as indicated). HMPV strain 001, SEQ ID NO: 12, is included in the alignments for comparison. Numbers at the left indicate the positional reference of the first residue in the partial amino acid sequence shown, and numbers to the right indicate the positional reference number of the terminal residue in the partial sequence. The more highly conserved or similar residues are denoted by shading. Dashes indicate a gap in the particular sequence introduced to maximize the alignment.

FIG. 27 is a graph illustrating multi-step growth of rHMPV-GFP containing the F456L mutation (see Figure 26A), compared to rHMPV, performed in LLC-MK2 cells as described above for Figure 13. Clones #3 and #7 represent viruses derived independently from two sister cDNA clones.

FIG. 28 is a diagram illustrating the GS signal of the M2 gene of wild type (WT) RSV (SEQ ID NO: 31), and a highly-attenuating T to C nucleotide substitution at position nine in the GS signal of the M2 gene of the attenuated cpts248/404 RSV mutant (Whitehead *et al.*, J. Virol. 72:4467-4471, 1998). There is a corresponding T residue that is highly conserved among all of the putative GS signals of HMPV. The individual GS motifs are named according to the putative ORF that each precedes (SEQ ID NO: 1, bases 41-57 for N; bases 1249-1265 for P; bases 2166-2182 for M; bases 3053-3069 for F; bases 4710-4726 for M2; bases 5467-5483 for SH; bases 6218-6234 for G; and bases 7119-7135 for L). The sequence at the bottom (SEQ ID NO: 32) indicates in upper case letters those positions in the putative HMPV GS signal that are exactly conserved among all of the signal in strain 83, which includes the T at position nine.

FIG. 29 is a diagrammatic representation of the genome structures of biologically-derived HMPV strains 83 and CAN98-75 (hereafter referred to as 75 or CAN75), representing the two proposed genetic HMPV subgroups and thus illustrating the diversity that must be considered in

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designing an HMPV immunogenic composition. Individual genes are indicated by boxes, with gene lengths and boundaries within the complete genomic sequence given in nucleotides together with the unmodified amino acid length (*italics*) of the deduced protein (in the case of M2, two predicted proteins, M2-1 and M2-2). The nucleotides lengths of the extragenic 3' leader, 5' trailer and intergenic regions are underlined.

FIG. 30 is a diagram illustrating the construction of chimeric viruses constructed with genes of HMPV and APV, which represent potential vaccine candidates. Part A illustrates the genome of HMPV83 and derivatives in which the N, P or M ORF is replaced individually by its APV counterpart (unshaded boxes). Parts B, C and D illustrate the structures of cDNAs containing the N, P and M genes, respectively of HMPV (upper line in each box) compared to a version containing the indicated substituted APV ORF (lower line in each box). Each ORF is shown as a shaded box and is flanked by HMPV GS (SEQ ID NO: 1, bases 12-57 for the leader/N GS signal; bases 1235-1265 for the N GE/P GS signal; and bases 2146-2182 for the P GE/M GS signal) and GE (SEQ ID NO: 1, bases 1235-1242 for the partial N GE signal; bases 2146-2151 for the partial P GE signal; and bases 3007-3037 for the M GE signal) transcription signals (boxed). In the case of the APV M gene, 64 nt of noncoding sequence that separates the ORF from the GE signal have been deleted (-64 nts nc). The GE and GS signals in turn are flanked by restriction sites selected from the following list, as indicated: *Bbs*I, *Bsm*BI, *Bfu*AI, *Bbs*I. Each of these sites consists of a recognition sequence (underlined) and has the property of cutting in a sequence-independent fashion at a second site outside of the recognition site (underlined tetranucleotide, bold) to leave a 4-nt 5'-protruding overhang. Here, the overhangs were TAAT or GTAG, as indicated, which matches the natural HMPV assignments at these positions. Part E illustrates a novel assembly strategy that utilizes the 4-nt overhangs, plus the flanking *Mlu*I and *Nhe*I sites, for ligation of the N, P and M cDNAs simultaneously into the HMPV backbone. By this strategy, this can be used to mix HMPV and APV N, P and M genes in whatever combinations desired to make replacements of single HMPV genes (as in part A) or to replace two or three genes simultaneously.

FIG. 31 is a diagram showing the comparison of the predicted gene boundaries, cis-acting GS and GE signals, and intergenic regions of HMPV strains 83 (SEQ ID NO: 1) and 75 (SEQ ID NO: 2), shown as positive-sense sequence. The top alignment shows the leader/N gene boundary, specifically the last 16-18 nucleotides of the leader region followed by the first 15 nucleotides of the N gene. The second alignment shows the N/P gene boundary, specifically the last 15 nucleotides of the N gene, followed by the adjoining intergenic region, followed by the first 15 nucleotides of the P gene. Subsequent alignments show the P/M, M/F, F/M2, M2/SH, SH/G, G/L, and L/trailer boundaries. Conserved sequence motifs at the end (gene end) and beginning (gene start) of each gene are indicated in bold upper case, and a consensus is given below (SEQ ID NOs: 34 and 35). Positions within these conserved motifs are numbered. Translational stop and start codons are underlined. Intergenic sequences are shown: in the case of the longer intergenic regions, only the

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first 5 nucleotides on the upstream and downstream ends are shown, and the number of nucleotides not shown is indicated. For each assignment in the consensus sequence, no more than two of the sequence pairs (HMPV 83 versus 75) could have a heterologous assignment in both subgroup sequences.

5 **FIG. 32** is a chart illustrating the percent amino acid and nucleotide sequence identity (the latter given in parentheses) between the predicted proteins and ORFs of: HMPV 83 versus HMPV 75, representing a comparison across subgroups; HMPV83 versus HMPV 00-1, representing a comparison within a subgroup; and RSV A2 versus RSV B1, representing a comparison across the two well-characterized RSV antigenic subgroups A and B, which serve here as a benchmark for assessing the magnitude and anticipated significance of the differences in the HMPV subgroups. Amino acid sequence identities were calculated based on the complete predicted proteins; in the case of G and SH, overhangs on the carboxy-terminal side of alignments due to length differences were not included in the calculations. Nucleotide sequence identities for the corresponding ORFs are shown in parentheses and are based on the protein-coding sequence exclusive of flanking noncoding sequence. For comparison, the HMPV intergenic regions were 48% identical between subgroups and noncoding gene sequences exclusive of conserved transcription signals were 54% identical, compared to values of 42% and approximately 50% for RSV (Johnson *et al.*, J. Gen. Virol. 69:2901-2906, 1988).

20 **FIGS. 33A-33B** are diagrams showing the alignment of the amino acid sequences of the SH (Figure 33A) and G (Figure 33B) proteins of HMPV strains 75 (SEQ ID NOs: 8 and 9, respectively), 83 (SEQ ID NOs: 5 and 6, respectively) and 00-1 (SEQ ID NOs: 10 and 11, respectively). This includes comparison between (75 versus 83) and within (83 versus 00-1) the proposed HMPV genetic subgroups. For strains 75 and 00-1, assignments that differ from that of 83 are shown; dashes indicate gaps introduced to maximize the alignment or to denote the absence of corresponding amino acids. Stars underneath each alignment denote amino acid identity among all three sequences; small dots indicate amino acid similarity among all three. Proposed signal/transmembrane domains are boxed. Motifs for N-linked carbohydrate are underlined (N-X-T/S, where X is not proline). In panel A, cysteine residues conserved among all three SH proteins are indicated with large dots, and potential sites for O-linked glycosylation (Hansen *et al.*, Glycoconj. J. 15:115-130, 1998) of the SH proteins are as follows: HMPV 83, 75, 77, 78, 81; HMPV 75, 78, 79, 81; 00-1, 77, 78, 81. The ectodomain of the G protein of each virus contained more than 40 potential acceptor sites for O-linked carbohydrate.

35 **FIG. 34** is a diagram illustrating the design of a chimeric HMPV virus bearing antigens specific to HMPV strains that represent two different antigenic subgroups of HMPV. The strain 83 antigenomic cDNA clone was used as the backbone (sequence representing part of the 3' leader region (bases 23-41 of SEQ ID NO: 1) and the putative N GS signal and beginning of the putative N ORF (bases 42-66 of SEQ ID NO: 1) is shown), and a transcription cassette was designed that

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consists of the ORF encoding the putative G, F, or SH surface proteins of strain 75 under the control of transcription signals from strain 83 (which including bases 42-57 of SEQ ID NO: 1, representing the conserved putative N GS signal, and bases 4685-4697 of SEQ ID NO: 1, representing the putative F GE signal). This transcription cassette is then inserted into the strain 83 backbone at a promoter-proximal site. This strategy for constructing a bivalent immunogenic construct employs the same strain 83 transcription signals and insertion site as shown for the HMPV-GFP construct in Figure 9.

FIG. 35 is a graph illustrating the daily level of replication of HMPV strains 75 and 83 in the upper and lower respiratory tract of African green monkeys. Mean daily virus titers in the nasopharyngeal (upper panel) or tracheal lavage (lower panel) specimens obtained on the indicated day post-inoculation from animals infected simultaneously by the intranasal and intratracheal routes with 10⁵ TCID₅₀ of the indicated virus: strain 75 (●), strain 83 (■).

FIGS. 36A-36B are a diagram and digitized images showing the construction and expression of a recombinant HPIV1 vector expressing the F protein of HMPV CAN83 (rHPIV1-F₈₃). The HPIV1 genome was modified by the creation of an *Mlu*I restriction site (HPIV1 nt 113-118) one nucleotide prior to the translational start codon of the N ORF (HPIV1 nt 119-121). The F ORF of HMPV strain CAN83 (1620 nt in length and encoding a 539 aa polypeptide; bases 3054-4697 of SEQ ID NO: 1) was engineered by PCR to be followed by the tetranucleotide TAAT and an HPIV1 gene junction consisting of a GE signal, CTT intergenic region, and a GS signal. The length of the entire cassette was 1656 nt and was designed, upon insertion into the *Mlu*I site, to conform to the rule of six and to maintain the HPIV1 GS signal sequence phasing. GS and GE signals are indicated (**FIG. 36A**). A portion of the predicted viral promoter that lies within the N gene is shown (**FIG. 36A**). LLC-MK2 cells were infected with rHPIV-F83, HMPV CAN83, rHPIV1 or mock-infected, as indicated, incubated for 72 hr, and analyzed by indirect immunofluorescence using anti-HMPV polyclonal hamster serum or a mixture of two mouse monoclonal antibodies to the HPIV1 HN protein, as indicated (**Fig. 36B**).

FIGS. 37A-37D are the complete sequence of the genome of biologically-derived strain 83, shown as positive sense DNA (SEQ ID NO: 1). See also, GenBank Accession No. AY297749.

FIGS. 38A-38D are the complete sequence of the genome of recombinant strain 83 bearing a transcription cassette expressing GFP, which recombinant strain exemplifies rHMPV-GFP of the current disclosure (SEQ ID NO: 3).

FIGS. 39A-39D are the complete sequence of the genome of biologically-derived, biologically-cloned HMPV CAN75 (SEQ ID NO: 2). See also, GenBank Accession No. AY297748.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the

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complementary strand is understood as included by any reference to the displayed strand. In the accompanying sequence listing:

- SEQ ID NO: 1 shows the nucleic acid sequence of the genome of biologically-derived
 5 HMPV (strain 83) as positive sense DNA.
 SEQ ID NO: 2 shows the nucleic acid sequence of the genome of biologically-derived
 HMPV (strain 75) as positive sense DNA.
 SEQ ID NO: 3 shows the nucleic acid sequence of the genome of recombinant HMPV
 (strain 83) with a transcription cassette expressing GFP as positive sense DNA.
 10 SEQ ID NO: 4 shows the amino acid sequence of the HMPV (strain 83) M2-1 protein.
 SEQ ID NO: 5 shows the amino acid sequence of the HMPV (strain 83) SH protein.
 SEQ ID NO: 6 shows the amino acid sequence of the HMPV (strain 83) G protein.
 SEQ ID NO: 7 shows the amino acid sequence of the HMPV (strain 83) L protein.
 SEQ ID NO: 8 shows the amino acid sequence of the HMPV (strain 75) SH protein.
 15 SEQ ID NO: 9 shows the amino acid sequence of the HMPV (strain 75) G protein.
 SEQ ID NO: 10 shows the amino acid sequence of the HMPV (strain 00-1) SH protein.
 SEQ ID NO: 11 shows the amino acid sequence of the HMPV (strain 00-1) G protein.
 SEQ ID NO: 12 shows the amino acid sequence of the HMPV (strain 00-1) L protein.
 SEQ ID NO: 13 shows the amino acid sequence of the HRSVA2 L protein.
 20 SEQ ID NO: 14 shows the amino acid sequence of the HPIV3 L protein.
 SEQ ID NO: 15 shows the amino acid sequence of the HPIV1 L protein.
 SEQ ID NO: 16 shows the amino acid sequence of the BPIV1 L protein.
 SEQ ID NO: 17 shows the nucleic acid sequence of the HMPV (strain 00-1) 3' leader.
 SEQ ID NO: 18 shows the nucleic acid sequence of the HMPV (strain 83) 3' leader.
 25 SEQ ID NO: 19 shows the nucleic acid sequence of the APV 3' leader.
 SEQ ID NO: 20 shows the nucleic acid sequence of the RSV 3' leader.
 SEQ ID NO: 21 shows the nucleic acid sequence of the HMPV (strain 00-1) 5' trailer.
 SEQ ID NO: 22 shows the nucleic acid sequence of the HMPV (strain 83) 5' trailer.
 SEQ ID NO: 23 shows the nucleic acid sequence of the APV 5' trailer.
 30 SEQ ID NO: 24 shows the nucleic acid sequence of the RSV 5' trailer.
 SEQ ID NO: 25 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus
 sequence.
 SEQ ID NO: 26 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus
 sequence, with alternative nucleic acid assignments.
 35 SEQ ID NO: 27 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus
 sequence, with alternative nucleic acid assignments.

SEQ ID NO: 28 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence.

SEQ ID NO: 29 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence, with alternative nucleic acid assignments.

5 SEQ ID NO: 30 shows the nucleic acid sequence of the HMPV (strain 83) GE consensus sequence, with alternative nucleic acid assignments.

SEQ ID NO: 31 shows the nucleic acid sequence of the RSV M2 GS sequence.

SEQ ID NO: 32 shows the nucleic acid sequence of the HMPV (strain 83) GS consensus sequence, with uniformly conserved bases.

10 SEQ ID NO: 33 shows the nucleic acid sequence of a polylinker used in the cloning and expression of rHMPV.

SEQ ID NO: 34 shows the nucleic acid sequence of the HMPV (strain 83/75) GE consensus sequence.

15 SEQ ID NO: 35 shows the nucleic acid sequence of the HMPV (strain 83/75) GS consensus sequence.

SEQ ID NO: 36 shows the nucleic acid sequence of the genome of HMPV (strain 00-1) as positive sense DNA.

20 DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

In one embodiment, methods and compositions are provided for producing an infectious, self-replicating, recombinant HMPV from one or more isolated polynucleotide molecules encoding viral sequences. The methods and compositions generally involve coexpressing in a cell or cell-free system one or more expression vectors comprising a polynucleotide molecule that encodes a partial or complete, recombinant HMPV genome or antigenome and one or more polynucleotide molecules encoding HMPV N, P, and L proteins, so as to produce an infectious HMPV particle. In certain embodiments, the methods and compositions for producing the recombinant HMPV further include expression of the M2-1 gene.

Typically, the polynucleotide molecule that encodes the recombinant HMPV genome or antigenome is a cDNA. Thus, disclosed herein are polynucleotides such as cDNAs and their equivalents that encode a recombinant HMPV. Expression vectors and constructs that incorporate a polynucleotide molecule encoding a recombinant HMPV genome or antigenome are also disclosed herein.

The HMPV genome or antigenome, and the N, P, M2-1 and L proteins can all be produced from a single expression vector. However, the genome or antigenome can be produced by a separate expression vector, and the N, P, M2-1 and L proteins can be produced by one, two, or more additional expression vectors. One or more of the N, P, M2-1 and L proteins can be supplied by

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expression of a recombinant HMPV genome or antigenome. These proteins can also be supplied by coinfection with the same or different HMPV. Thus, in several embodiments, one or more of the N, P, M2-1 and L proteins are from a heterologous HMPV.

5 Infectious, recombinant, self-replicating viral particles are disclosed that are produced according to the foregoing methods. These particles include complete viruses as well as viruses that lack one or more non-essential proteins or non-essential portions (for example, a cytoplasmic, transmembrane or extracellular domain) of a viral protein. Viruses of the current disclosure that lack one or more such non-essential components (for example, a gene or genome segment from the HMPV SH, G, M2-2 open reading frame (ORF) or comparable accessory ORFs, or a segment of one 10 or more other ORFs, or an intergenic or other non-coding or non-essential genome component) are referred to herein as incomplete viruses or "subviral particles." Exemplary subviral particles can lack any selected structural element, including a gene, gene segment, protein, protein functional domain, etc. that is present in a complete virus (that is, an assembled virion including a complete genome or antigenome, nucleocapsid and envelope). For example, a subviral particle of the current disclosure 15 can include an infectious nucleocapsid containing a genome or antigenome, and the products of the N, P, and L genes. Other subviral particles are produced by partial or complete deletions or substitutions of non-essential genes and/or their products among other non-essential structural elements.

Complete viruses and subviral particles produced by the methods disclosed herein are 20 typically infectious and self-replicative through multiple rounds of replication in a mammalian host amenable to infection by HMPV. These hosts include various *in vitro* mammalian cell populations, *in vivo* animal models widely known and accepted in the art as reasonably predictive of HMPV activity in humans (including, mice, hamsters, cotton rats, non-human primates including African green monkeys and chimpanzees), and humans, including seronegative and seropositive infants, 25 children, juveniles, and adults. However, viruses and subviral particles also can be produced that are highly defective for replication *in vivo*.

In one embodiment, the polynucleotide molecule encodes a sequence of a wild type HMPV (either the genome or the antigenome). The term "wild type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. 30 A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In another embodiment, the genome or antigenome can include one or more mutations from a biologically derived mutant HMPV, or any combination of recombinantly-introduced mutations; including one or more polynucleotide insertions, deletions, substitutions, or rearrangements that is/are selected to yield desired phenotypic 35 effects in the recombinant virus.

Thus, the recombinant HMPV genome or antigenome can be engineered using the methods disclosed herein to incorporate a recombinantly-introduced restriction site marker, or a translationally

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silent point mutation for handling or marking purposes. In other embodiments, the polynucleotide molecule encoding the recombinant HMPV genome or antigenome can incorporate one or more recombinantly-introduced attenuating mutations. In specific examples, the recombinant HMPV genome or antigenome incorporates one or more recombinantly-introduced, temperature sensitive (ts) or host range (hr) attenuating (att) mutations.

The recombinant HMPV genome or antigenome can incorporate one or more attenuating mutations identified in a biologically derived mutant HMPV strain, or in another mutant nonsegmented negative stranded RNA virus. For example, a mutation in a L, M, N, P, M2-1, M2-2, SH, F, or G protein, or in an extragenic sequence selected from a 3' leader or in a control signal such as the M2 GS sequence. Where the mutation is of one or more particular amino acid residues, the recombinant HMPV genome or antigenome can incorporate multiple nucleotide changes in the codons specifying the mutation to stabilize the modification against reversion.

The recombinant HMPV genome or antigenome can include an additional nucleotide modification specifying a phenotypic change, such as an alteration in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host-range restriction, or a change in immunogenicity. These additional modifications can alter one or more of the HMPV N, P, M, SH, G, F, M2-1, M2-2 and/or L genes and/or a 3' leader, 5' trailer, a cis-acting sequence such as a GS or GE sequence, and/or intergenic region within the HMPV genome or antigenome. For example, one or more HMPV genes can be deleted in whole or in part, or expression of the genes can be reduced or ablated by a frameshift mutation, by a mutation that alters a translation start site, by introduction of one or more stop codons in an ORF of the gene, or by a mutation in a transcription signal. In several embodiments, the recombinant HMPV genome or antigenome is modified by a partial or complete deletion of the HMPV SH, G, or M2-2 gene or ORFs, or one or more nucleotide changes that reduces or ablates expression of one or more HMPV genes yet yields a viable, replication competent, infectious viral construct. In other embodiments, the recombinant HMPV genome or antigenome is modified to encode a non-HMPV molecule such as a cytokine, a T-helper epitope, a restriction site marker, or a protein of a microbial pathogen capable of eliciting a protective immune response in a mammalian host.

A recombinant HMPV genome or antigenome can include a partial or complete HMPV "vector" genome or antigenome combined with one or more heterologous genes or genome segments (nucleic acid sequences) encoding one or more antigenic determinants of one or more heterologous pathogens to form a chimeric HMPV genome or antigenome. The heterologous genes or genome segments encoding the antigenic determinants can be added as supernumerary genes or genome segments adjacent to or within a noncoding region of the partial or complete HMPV vector genome or antigenome, or can be substituted for one or more counterpart genes or genome segments in a partial HMPV vector genome or antigenome. The heterologous genes or genome segments can include one or more heterologous coding sequences and/or one or more heterologous regulatory

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elements comprising an extragenic 3' leader or 5' trailer region, a GS signal, GE signal, editing region, translational start site, intergenic region, or a 3' or 5' non-coding region.

In additional embodiments, the heterologous pathogen is one or more heterologous pneumoviruses (for example, a heterologous HMPV or RSV) and the heterologous genes or genome segments encodes one or more HMPV or RSV N, P, M, SH, G, F, M2-1, M2-2 and/or L proteins or fragments thereof. Thus, the antigenic determinants can be from a heterologous HMPV or RSV G, SH and F glycoproteins, and antigenic domains, fragments and epitopes thereof, that is/are added to or substituted within the partial or complete HMPV genome or antigenome. In several examples, genes encoding G, SH and F glycoproteins of a heterologous HMPV or RSV are substituted for counterpart HMPV G, SH and F genes in a partial HMPV vector genome or antigenome. In additional examples, genes encoding the G, SH, and F glycoproteins of a heterologous HMPV or RSV are expressed in addition to the HMPV sequences. In this manner, a plurality of heterologous genes or genome segments encoding antigenic determinants of multiple heterologous pneumoviruses can be added to or incorporated within the partial or complete HMPV vector genome or antigenome. In other embodiments, one or more genes from APV or a related pneumovirus exhibiting a host range restriction in humans is used to replace the corresponding genes in humans to achieve an attenuated derivative and other improved properties.

The disclosed recombinant human metapneumoviruses (HMPVs) can be used to generate a desired immune response against one or more HMPVs, or against HMPV and one or more non-HMPV pathogens, in a subject susceptible to infection. Recombinant HMPV as disclosed herein are capable of eliciting a mono- or poly-specific immune response in an infected mammalian host, yet are sufficiently attenuated so as to not cause unacceptable symptoms of disease in the immunized host. The attenuated viruses, including complete viruses and subviral particles, can be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus can also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

Immunogenic compositions are also disclosed herein that include a physiologically acceptable carrier and/or adjuvant and an isolated attenuated recombinant HMPV virus. In one embodiment, the immunogenic composition includes a recombinant HMPV having at least one, at least two, or more attenuating mutations or other nucleotide modifications that specify a suitable balance of attenuation and immunogenicity. In specific examples, the immunogenic composition can be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The immunogenic composition can include an attenuated recombinant HMPV that elicits an immune response against a single HMPV strain or against multiple HMPV strains or serotypes or other pathogens such as RSV and/or HPIV. In this regard, recombinant HMPV can be combined in formulations with other HMPV strains, or with other candidate viruses such as a live attenuated RSV. Methods are also disclosed herein for stimulating the immune system of a mammalian subject to elicit an immune response against one or

more HMPVs, or against HMPV and a non-HMPV pathogen. Thus, a method is provided herein for inducing an immune response against a single HMPV, against multiple HMPVs, or against one or more HMPVs and a non-HMPV pathogen such as RSV.

5 The disclosed recombinant HMPVs can be used to elicit a monospecific immune response or a polyspecific immune response against multiple HMPVs, or against one or more HMPVs and a non-HMPV pathogen. Alternatively, recombinant HMPV having different immunogenic characteristics can be combined in a mixture or administered separately in a coordinated treatment protocol to elicit an immune response against one HMPV, against multiple HMPVs, or against one or more HMPVs and a non-HMPV pathogen such as RSV. In one example, the immunogenic compositions are
10 administered to the upper respiratory tract, for example, by spray, droplet or aerosol.

An operable set of viral open reading frames (ORFs) sufficient to direct viral transcription and RNA replication are disclosed herein. Diagnostic assays using this set of ORFs are provided that determine viral activities based either on infectious virus or on the expression of isolated polynucleotide molecules encoding viral sequences. In one example, an infectious HMPV
15 recombinant virus is disclosed herein that expresses a detectable marker or label, such as a recombinant HMPV expressing the jellyfish green fluorescent protein (GFP). These and related tools are effective, for example, to determine and characterize HMPV infection *in vitro* and *in vivo*. In one exemplary embodiment, recombinant HMPV expressing a detectable label can be utilized in assays and related compositions for the detection of HMPV-neutralizing antibodies in biological specimens
20 (for example, serum of patients at risk of HMPV infection or presenting with respiratory symptoms). In additional embodiments the recombinant HMPV expressing a detectable label can be used to screen compounds for antiviral activity.

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes VII*,
25 published by Oxford University Press, 2000 (ISBN 019879276X); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Publishers, 1994 (ISBN 0632021829); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by Wiley, John & Sons, Inc., 1995 (ISBN 0471186341); and other similar references.

30 As used herein, the singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Also, as used herein, the term "comprises" means "includes." Hence "comprising A or B" means including A, B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids
35 or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications,

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patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

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As described above, methods and compositions for the production and use of novel, recombinant HMPVs are provided herein. The recombinant HMPVs are infectious and immunogenic in humans and other mammals and are useful as immunogenic compositions to produce immune responses against one or more HMPVs or other pneumoviruses. Thus, the recombinant HMPVs can be used to produce an immune response to, for example, RSV and/or one or more HMPV or other pneumoviral strains, serotypes, or subgroups. Chimeric HMPVs are provided herein that can be used to elicit an immune response against a selected HMPV and one or more additional pathogens, for example against multiple HMPVs or against a HMPV and a non-HMPV virus such as RSV, or parainfluenza virus (PIV). The immune response elicited can involve either or both humoral and/or cell mediated responses. The HMPVs can be attenuated to yield a desired balance of attenuation and immunogenicity.

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Methods are provided herein for designing and producing attenuated, HMPVs that are useful as agents for eliciting a desired immune response against HMPV and other pathogens. Thus, using the methods disclosed herein, recombinant HMPVs can be produced that have a defined genome sequence and predictable characteristics.

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An exemplary recombinant HMPV (rHMPV) includes a recombinant HMPV genome or antigenome and encodes (or the antisense of which encodes) HMPV major nucleocapsid (N) protein, nucleocapsid phosphoprotein (P), and large polymerase protein (L) (see FIG. 1 and exemplary sequence information provided in FIG. 37). In additional embodiments, the rHMPV can incorporate a recombinant HMPV genome or antigenome, N, P, and L proteins, and a HMPV M2-1 protein. In further embodiments, one or more of the N, P, L, and/or M2-1 proteins can be a mutant protein or partial protein, or include a corresponding homologous protein or protein fragment of a heterologous HMPV or non-HMPV virus such as APV. One or more additional HMPV proteins can be coexpressed with the recombinant HMPV genome or antigenome, in various combinations, to provide a range of infectious viruses. As used herein the term "recombinant HMPV" or "rHMPV" includes recombinantly produced subviral particles lacking one or more non-essential viral components, complete viruses having all native viral components, and viruses containing supernumerary proteins, antigenic determinants or other additional components. A recombinant virus is produced entirely from cloned cDNA.

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As set forth in the Examples below, a complete consensus sequence was determined for the genomic RNA of an exemplary wild type strain of HMPV. The sequence thus identified was used to generate a full-length antigenomic cDNA and to recover a wild type rHMPV. The biological

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properties of rHMPV *in vitro* and *in vivo* demonstrates that the exemplary rHMPV sequence corresponds to a wild type virus. This critical finding demonstrates that the recombinant HMPV sequence disclosed herein is that of an authentic wild type virus. This rHMPV serves as a novel substrate for recombinant introduction of attenuating mutations for the generation of live-attenuated HMPV and HMPV-based chimeric and vectors that can be used for immunogenic compositions.

As disclosed herein, infectious recombinant HMPVs can be produced by a recombinant coexpression system that permits introduction of defined changes into the recombinant HMPV and provides for the generation, with high frequency and fidelity, of HMPV having a defined genome sequence. These modifications are useful in a wide variety of applications, including the development of live attenuated viral strains bearing predetermined, defined attenuating mutations. Infectious HMPV can be produced by intracellular or cell-free coexpression of one or more isolated polynucleotide molecules that encode the HMPV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins to generate a transcribing, replicating nucleocapsid. An "infectious virus" is a viral particle that has the ability to deliver its genome to the cytoplasm of a host cell.

The cDNAs encoding a HMPV genome or antigenome are constructed for intracellular or *in vitro* coexpression with the selected viral proteins to form infectious HMPV. A "HMPV antigenome" is an isolated positive-sense polynucleotide molecule which serves as a template for synthesis of progeny HMPV genome. In one embodiment a cDNA is constructed which is a positive-sense version of the HMPV genome that corresponds to the replicative intermediate RNA, or antigenome. This minimizes the possibility of hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid. A "replication competent virus" is a viral particle capable of initiating an infection that produces viral progeny.

In some embodiments the genome or antigenome of a recombinant HMPV contains only those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, that is, a gene may be provided by complementation or the like from a separate nucleotide molecule. In other embodiments, the HMPV genome or antigenome encodes all functions necessary for viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

A "recombinant HMPV particle" is a HMPV or HMPV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced there from. The recombinant expression system can employ a recombinant expression vector which includes an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory role in HMPV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into HMPV RNA, and appropriate

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transcription initiation and termination sequences. A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence.

5 To produce infectious HMPV from a cDNA-expressed HMPV genome or antigenome, the genome or antigenome is coexpressed with those HMPV or heterologous viral proteins necessary to produce a nucleocapsid capable of RNA replication, and render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other HMPV proteins and initiates a productive infection. Alternatively, additional HMPV proteins useful for a productive infection can be supplied by coexpression.

10 In certain embodiments, complementing sequences encoding proteins necessary to generate a transcribing, replicating HMPV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. In one embodiment, the helper virus can be distinguished phenotypically from the virus encoded by the HMPV cDNA. For example, it may be desirable to provide monoclonal antibodies that react immunologically with the helper virus but not the virus encoded by the HMPV cDNA. Such antibodies can be neutralizing antibodies. In some
15 embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the HMPV cDNA to provide antigenic diversity from the helper virus, such as in the HMPV glycoprotein genes.

20 Expression of the HMPV genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a selected promoter (for example, for T7 RNA polymerase), which in turn is supplied by infection, transfection or transduction with a suitable expression system (for example, for the T7 RNA polymerase, such as a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase, as described by
25 Wyatt *et al.*, Virology 210:202-205, 1995). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells (see Buchholz *et al.*, J. Virol. 73:251-259, 1999) or by transfection of preformed mRNA or protein.

A HMPV genome or antigenome can be constructed for use by, for example, assembling cloned cDNA segments, representing in aggregate the complete genome or antigenome, by PCR or
30 the like (described in, for example, U.S. Patent Nos. 4,683,195 and 4,683,202, and PCR Protocols: A Guide to Methods and Applications, Innis *et al.*, eds., Academic Press, San Diego, 1990) of reverse-transcribed copies of HMPV mRNA or genome RNA. For example, a first construct can be generated which includes cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (for example, T7 RNA polymerase promoter) and assembled in an appropriate
35 expression vector, such as a plasmid, cosmid, phage, or DNA virus vector. The vector can be modified by mutagenesis and/or insertion of a synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired HMPV proteins

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can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and single or tandem T7 transcriptional terminators. The ribozyme can be hammerhead type, which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta *et al.*, Nature 350:434-436, 1991) that would yield a 3' end free of non-PIV nucleotides.

Alternative means to construct cDNA encoding the HMPV genome or antigenome include RT-PCR using different PCR conditions (for example, as described in Cheng *et al.*, Proc. Natl. Acad. Sci. USA 91:5695-5699, 1994) to reduce the number of subunit cDNA components to as few as one or two pieces. In other embodiments different promoters can be used (for example, T3, SPQ or different ribozymes, such as that of a hammerhead variety. Different DNA vectors (for example, cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

By "infectious clone" or "infectious cDNA" of HMPV is meant cDNA or its product, synthetic or otherwise, as well as RNA capable of being directly incorporated into infectious virions which can be transcribed into genomic or antigenomic HMPV RNA that can serve as a template to produce the genome of infectious HMPV viral or subviral particles. As noted above, defined mutations can be introduced into an infectious HMPV clone by a variety of conventional techniques (for example, site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA constructs provide for better ease of manipulation than large cDNA constructs, and then readily assembled into a complete cDNA.

Isolated polynucleotides (for example, cDNA) encoding the HMPV genome or antigenome can be introduced into cells that can support a productive HMPV infection by transfection, electroporation, mechanical insertion, transduction or the like. Exemplary cells of use are HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, baby hamster kidney (BHK), and Vero cells. Isolated polynucleotide sequences can be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler *et al.*, Cell 14:725, 1978; Corsaro *et al.*, Somatic Cell Genetics 7:603, 1981; Graham *et al.*, Virology 52:456, 1973, electroporation (Neumann *et al.*, EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel *et al.*, (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), cationic lipid-mediated transfection (Hawley-Nelson *et al.*, Focus 15:73-79, 1993) or a commercially available transfection reagent, for example, Lipofectamine-2000 (Invitrogen, Carlsbad, CA) or the like.

By providing infectious clones of HMPV, a wide range of alterations can be recombinantly produced within the HMPV genome (or antigenome), yielding defined mutations that specify desired phenotypic changes. The compositions and methods disclosed herein for producing recombinant HMPV permit ready detailed analysis and manipulation of HMPV molecular biology and pathogenic

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mechanisms using, for example, defined mutations to alter the function or expression of selected HMPV proteins. Using these methods and compositions, one can readily distinguish mutations responsible for desired phenotypic changes from silent incidental mutations, and select phenotype-specific mutations for incorporation into a recombinant HMPV genome or antigenome. In this context, a variety of nucleotide insertions, deletions, substitutions, and rearrangements can be made in the HMPV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme sites. Alternatively, such techniques as site-specific mutagenesis, alanine scanning mutagenesis, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Recombinant modifications of HMPV can be directed toward the production of improved candidate viruses, for example, to enhance viral attenuation and immunogenicity, to ablate epitopes associated with undesirable immunopathology, to accommodate antigenic drift, etc. To achieve these and other objectives, the compositions and methods disclosed herein allow for a wide variety of modifications to be introduced into a HMPV genome or antigenome for incorporation into infectious, recombinant HMPV. For example, foreign genes or gene segments encoding antigenic determinants (for example, protective antigens or immunogenic epitopes) can be added within a HMPV clone to generate recombinant HMPVs capable of inducing immunity to both HMPV and another virus or pathogenic agent from which the antigenic determinants was/were derived. Alternatively, foreign genes can be inserted, in whole or in part, encoding modulators of the immune system, such as cytokines, to enhance immunogenicity of a candidate virus. Other mutations that can be included within HMPV clones are, for example, substitution of heterologous genes or gene segments (for example, a gene segment encoding a cytoplasmic tail of a glycoprotein gene) with a counterpart gene or gene segment in a HMPV clone. Alternatively, the relative order of genes within a HMPV clone can be changed, a HMPV genome promoter or other regulatory element can be replaced with its antigenome counterpart, or selected HMPV genes rendered non-functional (for example, by functional ablation involving introduction of a stop codon to prevent expression of the gene). In addition, the codon selection of genes such as those encoding the major protective antigens can be modified to improve the efficiency of translation. Other modifications in a HMPV clone can be made to facilitate manipulations, such as the insertion of unique restriction sites in various non-coding or coding regions of the HMPV genome or antigenome. In addition, nontranslated gene sequences or intergenic regions can be shortened or removed to increase capacity for inserting foreign sequences.

As noted above, it is often desirable to adjust the phenotype of recombinant HMPVs for use by introducing additional mutations that attenuate the virus, affect the virulence of the virus, or otherwise alter the phenotype of the recombinant virus. One of skill in the art can readily identify methods and procedures for mutagenizing, isolating and characterizing HMPV to obtain attenuated mutant strains (for example, temperature sensitive (*ts*), cold passaged (*cp*), cold-adapted (*ca*), small

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plaque (*sp*) and host-range restricted (*hr*) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype (see, for example, Durbin *et al.*, Virology 235:323-332, 1997; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997). Thus, methods are provided herein for determining replication, immunogenicity, genetic stability and immunogenic efficacy of biologically derived and recombinantly produced attenuated HMPVs in accepted model systems reasonably correlative of human activity, including hamster or rodent and non-human primate model systems.

In additional embodiments, rHMPV candidates are constructed by introduction of nucleotide or amino acid point mutations that confer attenuation or other desired phenotypes. Such mutations can involve substitution of one or more nucleotides or amino acids at a given locus, or can involve small deletions in which one or more nucleotides or amino acids at a given locus are deleted. These mutations are termed "point mutations" here to denote that each particular mutation is circumscribed. For example, a typical mutation can involve changing a single amino acid by substituting 1, 2 or 3 nucleotides in the corresponding codon. As another example, the three nucleotides might be deleted altogether, resulting in the deletion of a single amino acid in the encoded protein. Point mutations can be identified empirically, such as by systematically replacing charged amino acids in one or more of the HMPV proteins with ones that are uncharged, for example, replacing aspartate, glutamate, lysine or arginine with alanine.

Alternatively, attenuating amino acid substitutions and other mutations can be devised using existing mononegavirus mutations as a guide, for example mutations in a different "biologically derived" HMPV, non-human pneumovirus, such as an APV, or non-HMPV virus, such as a RSV or PIV. By "biologically derived" is meant any virus not produced by recombinant DNA methods. Thus, biologically derived HMPV include all naturally occurring HMPVs, including, for example, naturally occurring HMPV having a wild type genomic sequence and HMPV having allelic or mutant genomic variations from a reference wild type HMPV sequence, for example, HMPV having a mutation specifying an attenuated phenotype. Likewise, biologically derived HMPV include HMPV mutants derived from a parental HMPV by, inter alia, artificial mutagenesis and selection procedures not involving direct recombinant DNA manipulation. Attenuating mutations in biologically derived HMPV and other nonsegmented negative stranded RNA viruses for incorporation within recombinant HMPV can occur naturally or can be introduced into wild type HMPV strains and thereafter identified and characterized by well known mutagenesis and analytic procedures. For example, incompletely attenuated parental HMPV or other heterologous viral mutant strains can be produced by chemical mutagenesis during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperatures in order to

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introduce growth restriction mutations, or by selection of a mutagenized virus that produces small plaques (sp) in cell culture. In addition, known mutations in existing mononegaviruses that have been produced by recombinant methods also can serve as a guide for devising desired mutations in HMPV. Such known mutations in heterologous viruses can be ones that had been deliberately introduced by directed mutagenesis, or can be ones that arose spontaneously during the process of DNA manipulation and virus recovery and propagation, or can be ones that arose in recombinantly derived viruses that were exposed subsequently to mutagenic agents or passaged in a manner designed to favor phenotypic changes, such as described above for generating mutations in biologically derived viruses.

In certain embodiments, the HMPV genome or antigenome is recombinantly modified to incorporate an attenuating mutation at an amino acid position corresponding to an amino acid position of an attenuating mutation identified in a heterologous, mutant nonsegmented negative stranded RNA virus. The virus can be either biologically derived or of recombinant origin. Based on routine sequence alignments and other analyses, mutations previously identified in a heterologous HMPV or non-HMPV virus are mapped to a corresponding position in HMPV for "transfer" (that is, introduction of an identical, conservative or non-conservative mutation, potentially including a substitution, deletion or insertion, at a homologous or corresponding position identified by the alignment) into recombinant HMPV. In many cases, an alignment of the two sequences of interest is sufficient to identify the corresponding residue in HMPV, particularly if the percent amino acid identity is substantial (for example, approximately 35-40% identity or greater) or high (for example, 70% identity or greater, such as at least about 80%, 90%, 95%, or 99% identity). In other cases, particularly when the amino acid relatedness is not substantial (for example, less than approximately 35% identity), additional heterologous related viruses can be included in the alignment in order to obtain reliably identify conserved residues that serve as markers to identify corresponding positions. In one embodiment, the percentage of sequence identity associated with the terms "substantial" or "high" or "not high" are used herein in the context of comparison of heterologous viruses from different serotypes or taxonomic groups.

A large assemblage of such candidate mutations are available for transfer into rHMPV of the current disclosure (see, for example, (Durbin *et al.*, Virology 235:323-332, 1997; Skiadopoulos *et al.*, J. Virol. 72:1762-1768, 1998; Skiadopoulos *et al.*, J. Virol. 73:1374-1381, 1999; Skiadopoulos *et al.*, Virology 260:125-35, 1999; Durbin *et al.*, Virology 261:319-30, 1999; Newman *et al.*, Virus Genes 24:77-92, 2002; Feller *et al.*, Virology 276:190-201, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078); U.S. Provisional Application No. 60/059,385, filed September 19, 1997; U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No.

60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002).

Within these embodiments, it is often desired to modify the recipient recombinant HMPV genome or antigenome to encode an alteration at the subject site of mutation that corresponds conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild type sequence, then a similar substitution can be engineered at the corresponding residues in the recombinant HMPV. In one example, the substitution will specify an identical or conservative amino acid to the substitute residue present in the mutant viral protein (see below for a description of conservative substitutions). However, it is also possible to alter the native amino acid residue at the site of mutation non-conservatively with respect to the substitute residue in the mutant protein (for example, by using any other amino acid to disrupt or impair the function of the wild type residue). Negative stranded RNA viruses from which exemplary mutations are identified and transferred into a recombinant HMPV of the current disclosure include heterologous strains of HMPV, other non-HMPV pneumoviruses (for example, human RSV, bovine RSV, APV, pneumonia virus of mice (PVM)), PIVs (for example, HPIV1, HPIV2, HPIV3, BPIV3, and MPIV1), Newcastle disease virus (NDV), simian virus 5 (SV5), measles virus (MeV), rinderpest virus, canine distemper virus (CDV), rabies virus (RaV), and vesicular stomatitis virus (VSV), among others.

As depicted in Figures 26A-26F, one or more corresponding sites of mutation that specify a desired phenotypic change in a heterologous virus (for example, when indicated wild type residues at the designated positions is/are altered, for example, by substitution) is identified by conventional sequence alignment. The corresponding target sites for mutation (see, for example, boxed sites in Figures 26A-26F) in HMPV is thereby mapped for transfer of the mutation into a recombinant HMPV to yield attenuation or other desired phenotypic changes. More specifically, corresponding amino acids between the compared heterologous mutant and HMPV sequences represent target sites for identical or conservative transfer (for example, by recombinant engineering involving site-directed mutagenesis of a HMPV antigenomic cDNA) of the subject mutation into a recombinant HMPV. In some embodiments, attenuating and other desired mutations identified in one negative stranded RNA virus are thereby targeted for transfer (for example, to be copied identically or conservatively by substitution mutagenesis) into a corresponding position within the genome or antigenome of a recombinant HMPV of the current disclosure. Related methods for rational design of other recombinant mutant RNA viruses are described, for example, in International Application No. PCT/US00/09695, filed April 12, 2000, published as WO 00/61737 on 10/19/00 corresponding to U.S. National Phase application 09/958,292, filed on 01/08/02, and claiming priority to U.S. Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999. Additional description pertaining to this aspect of the current disclosure is provided in Newman *et al.*, Virus Genes 24:77-

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92, 2002; Feller *et al.*, Virology 10;276:190-201, 2000; Skiadopoulos *et al.*, Virology 260:125-35, 1999; and Durbin *et al.*, Virology 261:319-30, 1999.

In one exemplary embodiment, amino acid sequence alignments are made between the L gene of HMPV and the L genes of other mononegaviruses for which one or more attenuating
5 substitutions in the L protein have been identified. This embodiment of the current disclosure is illustrated in FIGS. 26A-26F, which depict sequence alignments of various segments of the putative L protein of HMPV with corresponding segments of L proteins of a number of other mononegaviruses in which attenuating mutations have been mapped. As exemplified in FIGS. 26A-26F, various mutations identified in the L gene of a heterologous negative stranded RNA virus can be
10 incorporated into recombinant HMPV to yield attenuation or other desired phenotypic changes. These figures provide exemplary, partial L gene sequence alignments between an HMPV wild type virus and the indicated viruses (including as examples, a different HMPV, RSV, HPIV1 HPIV3, and BPIV3). The examples of attenuating mutations include ones identified in viruses of biological or recombinant origin. The alignments are employed to identify regions containing known attenuating
15 mutations in the heterologous virus.

FIGS. 26A-26F illustrate the identification and mapping of various exemplary attenuating mutations in the L gene for transfer into rHMPV (note that the numbers at the left indicate the positional reference number of the first residue in the partial sequence shown, and the numbers at the right indicate the positional reference number of the terminal residue in the partial sequence). FIG.
20 26A shows a Phenylalanine-521 to Leucine (F521L) substitution in the L protein specified by a mutation in the L gene of an RSV cold-passage temperature-sensitive (cpts) mutant cpts530 (Juhasz *et al.*, J. Virol. 71:5814-9, 1999). Also shown is an attenuating double mutation of Arginine-588-Alanine (R588A) and Aspartate-589-Alanine (D589A) that was originally developed in RSV (Tang *et al.*, Virology 302:207-16, 2002). FIG. 26B shows the positions of attenuating Tyrosine-942-Histidine (Y942H) and Leucine-992-Tyrosine (L992Y) mutations identified in the PIV3 cp45 vaccine
25 candidate (Skiadopoulos *et al.* J. Virol. 72:31762-8, 1998; U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002). FIG. 26C shows the positions of the attenuating Isoleucine-1103-Valine (I1103V) mutation originally identified
30 in BPIV3 (Haller *et al.*, Virology 288:342-50, 2001), the attenuating Threonine-1558-Isoleucine (T1558I) mutation identified in PIV3 cp45 (Skiadopoulos *et al.*, J. Virol. 72:31762-8, 1998), and the Cysteine-319-Tyrosine (C319Y) mutation identified the attenuated cpRSV vaccine candidate (Connors *et al.*, Virology 208:478-84, 1995). FIG. 26D shows the positions of the attenuating Glutamine-831-Leucine (Q831L) mutation identified in cpts248 RSV (Firestone *et al.*, Virology
35 225:419-22, 1996), the attenuating Methionine-1169-Valine (M1169V) mutation identified in the RSV mutant cpts530/109 (Juhasz *et al.*, J. Virol. 73:5176-80), the Aspartate-1183-Glutamate (D1183) mutation identified in the RSV mutant cpts248/404 (Firestone *et al.*, Virology 225:419-22,

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1996), and the combination of six point mutations that constitute the attenuating C9 cluster characterized in recombinant RSV (Tang *et al.*, Virology 302:207-16, 2002): Aspartate-1187-Alanine (D1187A), Lysine-1188-Alanine (K1188A), Arginine-1189-Alanine (R1189A), Glutamate-1190-Alanine (E1190A), Glutamate-1208-Alanine (E1208A), and Arginine-1209-Alanine (R1209A). FIG. 26E shows the position of the attenuating Tyrosine-1321-Asparagine (Y1321N) mutation of RSV cpts530/1030 (Whitehead *et al.*, J. Virol. 73:871-7, 1999), and the Histidine-1690-Tyrosine (H1690Y) mutation of the attenuated cpRSV derivative (Connors *et al.*, Virology 208, 478-84, 1995). FIG. 26F shows the positions of an attenuating Asparagine-43-Isoleucine (N43I) mutation identified in the cpts RSV mutant 248/955, and the attenuating Threonine-1711-Isoleucine (T1711I) mutation characterized in the chimeric virus rHPIV3-L_B, consisting of HPIV3 in which the L gene was replaced by that of BPIV3 (Skiadopoulos *et al.*, J. Virol. 77:1141-8, 2003).

The foregoing alignments show, for example, that the F521 residue in the RSV sequence is exactly conserved among most of the other mononegaviruses examined (23 out of 24 paramyxoviruses and 1 out of 3 rhabdoviruses) and, in particular, is present in HMPV. It should be noted that the amino acid position of the corresponding residue is not the same in each L protein, reflecting differences in length as well as small deletions and insertions elsewhere in the various individual molecules. Thus, the corresponding residue is F456 in HMPV. From this comparative mapping analysis, the F456 residue in HMPV is identified as a target for either an identical, conservative, or even non-conservative amino acid substitution (for example, substitution of the F456 residue by a leucine, or by a conservative or non-conservative amino acid as compared to leucine). The same alignment (FIG. 26A) shows that the D589 assignment is also exactly conserved in each of sequences. The R588 assignment is not conserved in each, but it is represented by the homologous assignment of Lysine in HMPV. With respect to the attenuating Tyrosine-942-Histidine (Y942H) and Leucine-992-Tyrosine (L992Y) mutations of PIV3 cp45, in both of these instances the exact amino acid assignment was not conserved, but the presence of various highly-conserved residues throughout the alignment show that these positions correspond (Figure 26B). Positional correspondence in this context indicates that the aligned, corresponding target site for mutation (whether it is occupied by an amino acid that is identical, conservative or divergent in structural character compared the corresponding residue in the wild type (for example, a non-attenuated parent) sequence of the heterologous virus) will often yield an attenuation or other desired phenotype in a rHMPV upon mutation from the HMPV wild type sequence to a different residue (that is, to a residue identity that is the same, conservative, or even distinct structurally in comparison to the corresponding residue in the heterologous mutant).

In addition to the foregoing L gene mutations, two temperature sensitive (ts) mutations that were reported in the P protein of recombinant RSV, namely Glycine-172-Serine and Glutamate-176-Glycine (Lu *et al.*, J. Virol. 76:2871-80, 2002), can be transferred to yield a predicted attenuation phenotype in a rHMPV P, by altering the corresponding one or both of the HMPV residues Glycine-

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213 and Glutamate-217, respectively. Likewise, a mutation in the N protein of the attenuated derivative cpRSV, specifying a Valine-267-Isoleucine substitution (Connors *et al.* Virology 208:478-84, 1996), maps to Serine-268 in HMPV, whereby an Ile or other, conservative or non-conservative, substitution of this residue in a rHMPV can yield useful candidates for identification of additional
5 attenuated derivatives. In the same manner, two mutations in the F protein of cpRSV, namely Glutamate-218-Alanine and Threonine-523-Isoleucine (Connors *et al.* Virology 208:478-84, 1996), correspond to Lysine-188 and Threonine-489, respectively, in the HMPV F protein, which therefore represent target sites for introduction of an attenuating amino acid substitution in rHMPV.

10 In other embodiments, mutations in a cis-acting regulatory sequence or other non-coding sequence are identified in a heterologous virus (including biologically or otherwise derived mutant HMPV of the same or different strain, subtype, species, RSV, PIV, APV, etc.) and incorporated in a rHMPV of the current disclosure. For example, the identification of the GS and GE motifs identifies these as targets for mutagenesis. The short, circumscribed nature of these signals is amenable to a systematic evaluation of the effects of mutations at each position. Mutations in RNA signals such as
15 these can be readily assayed in a mini-replicon system as well as in complete virus.

FIG. 28 shows an example of transferring a mutation that involves a cis-acting RNA signal. A potent attenuating point mutation identified for the cpts 248/404 derivative of RSV involves a point mutation in the GS signal of the M2 gene: specifically, the T residue (positive sense) at position nine was changed to C (see, for example, Whitehead *et al.*, Virology 247:232-9, 1998). In the proposed
20 GS signal of HMPV, a T residue at position nine is conserved in each of the proposed HMPV genes (FIG. 28). This substitution can be made in any of the HMPV genes.

In accordance with the foregoing description, a large panel of desired mutations identified in heterologous viruses can be "transferred" into a rHMPV, even in cases of low sequence relatedness. In these embodiments, there is not a strict requirement that the corresponding wild type or final
25 mutant amino acid assignment be identical to that of the heterologous virus. Also, rather than an amino acid substitution, it is possible to delete one or several amino acids or, alternatively, insert one or several amino acids. In designing substitution mutations for HMPV, a useful strategy is to evaluate all possible alternative amino acid assignments at this position. This is a useful method for evaluating the full range of possible attenuation phenotypes involving this position, and can produce
30 mutants exhibiting a range of temperature sensitivity and attenuation (see, for example, U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002). In addition, the choice of codon for substitution can be made to involve the greatest number of nucleotide differences relative to both the wild type and to any possible alternative
35 amino acid assignments that are found to not yield attenuated phenotypes. This has the effect of reducing the probability of reversion or mutation to lose the attenuated phenotype. Specifically, it is estimated that the frequency of reversion at any single nucleotide position is 10^{-4} to 10^{-5} , and hence

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the frequency of reversion/mutation at two nucleotide positions is 10^{-8} to 10^{-10} , and the frequency of reversion/mutation involving three nucleotides is 10^{-12} to 10^{-15} . Thus, point mutations involving multiple nucleotide substitutions can greatly increase genetic and phenotypic stability. Also, the mutation can be designed to involve deletion of a single amino acid or more than one amino acid, which can reduce the possibility of reversion.

It should be noted that the mutations illustrated in FIGS. 26A-26F are only exemplary by virtue that the depicted mapping exercise was limited to examples involving amino acid changes in the L protein, whereas the same strategy can be applied in accordance with the tools and description provided herein to transfer attenuating mutations involving any viral protein. For example, two *ts* mutations that were developed in the P protein of recombinant RSV, namely Glycine-172-Serine and Glutamate-176-Glycine (Lu *et al.*, J. Virol. 76:2871-80, 2002), correspond to HMPV P residues Glycine-213 and Glutamate-217, respectively. A mutation in the N protein of the attenuated derivative *cp*RSV, namely Valine-267-Isoleucine (Connors *et al.* Virology 208:478-84, 1996), corresponds to Serine-268 in HMPV. Also, two mutations in the F protein of *cp*RSV, namely Glutamate-218-Alanine and Threonine-523-Isoleucine (Connors *et al.* Virology 208:478-84, 1996), correspond to Lysine-188 and Threonine-489, respectively, in the HMPV F protein. Thus, any known mutation having a desirable phenotypic property present in any nucleotide or amino acid sequence is a useful candidate for incorporation in rHMPV by mutation of corresponding residues in a rHMPV RNA or protein employing the novel tools and methods of the current disclosure.

Another method for devising attenuating or otherwise desirable amino acid substitution mutations involve "charge-to-alanine" or "alanine scanning" mutagenesis where, for example, one or more charged amino acid residues are each changed to alanine residues (Wertman *et al.*, Genetics 132:337-50, 1992; Hassett and Condit, Proc. Natl. Acad. Sci. USA 91:4554-58, 1994; Tang *et al.*, Virology 302:207-16, 2002. A typical target is a pair of residues, but residues can be changed singly or in larger combinations. It is often useful to systematically change ("scan") residues across an encoded protein. Any protein can be a target, although the L protein is the most common locus of mutations in attenuated mononegaviruses. Alternatively, one advantage of this strategy is that one can target proteins that are not represented in available mutants. This strategy can be readily addressed with the methods disclosed herein. In particular, the minireplicon system offers a method for screening mutations in the N, P, L and M2-1 proteins. In that strategy, mutations are introduced into the appropriate support plasmid, such as the one encoding the L protein, and evaluated for the ability to direct the replication and transcription of a minigenome containing a reporter gene. For example, the assay can be done in replica at 32°C and 37°C (or any temperature in the range of 35°C -41°C) to identify *ts* mutations that operate at the lower, permissive temperature and are inhibited at the higher temperature. This offers a more rapid and technically simple method for performing an initial screening, after which appropriate mutations can be introduced into complete recombinant virus.

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Thus, the methods disclosed herein can be used to develop mutations that confer improved properties to HMPV for the purposes of developing an immunogenic composition or for other purposes, such as purifying viral proteins. Mutations can be optimized, such as by evaluating all possible amino acid or nucleotide assignments at a given position, or by making amino acid changes
5 involve codon choices that will be less likely to revert to wild type or to an alternative assignment that yields a wild type phenotype. Other changes can be made that fundamentally alter the viral backbone, such as changes in gene order or movement of major protective antigen genes to a promoter-proximal position. Importantly, these various modifications can be introduced into rHMPV in combination to develop viruses for use in immunogenic compositions or for other purposes. The
10 methods described herein allows for the systematic modification of viruses as necessary, such as the introduction of additional attenuating mutations into a recombinant virus in response to clinical studies.

As noted above, production of a sufficiently attenuated biologically derived HMPV or other viral mutant can be accomplished by several known methods. One such procedure involves
15 subjecting a partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, partially attenuated mutants are produced by passage in cell cultures at suboptimal temperatures. Thus, a cold-adapted (*ca*) mutant or other partially attenuated HMPV strain is adapted to efficient growth at a lower temperature by passage in culture. This selection of mutant HMPV during cold-passages substantially reduces any residual virulence in the derivative strains as
20 compared to the partially attenuated parent. Alternatively, specific mutations can be introduced into biologically derived HMPV by subjecting a partially attenuated parent virus to chemical mutagenesis, for example, to introduce *ts* mutations or, in the case of viruses which are already *ts*, additional *ts* mutations sufficient to confer increased attenuation and/or stability of the *ts* phenotype of the attenuated derivative. Means for the introduction of *ts* mutations into HMPV include replication of
25 the virus in the presence of a mutagen such as 5-fluorouridine according to generally known procedures. Other chemical mutagens can also be used. Attenuation can result from a *ts* mutation in almost any HMPV gene, although a particularly amenable target for this purpose has been found to be the polymerase (L) gene. The level of temperature sensitivity of replication in exemplary attenuated HMPV can be determined by comparing its replication at a permissive temperature with
30 that at several restrictive temperatures. The lowest temperature at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed the "shutoff temperature." In experimental animals and humans, both the replication and virulence of HMPV correlate with the mutant's shutoff temperature.

From biologically and recombinantly derived HMPV and other nonsegmented negative
35 stranded RNA viruses, a large "menu" of attenuating mutations is identifiable by the teachings herein, each of which can be combined with any other mutations for adjusting the level of attenuation, immunogenicity and genetic stability in recombinant HMPV. In this context, many recombinant

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HMPV candidates will include one or more, and preferably two or more, mutations from a biologically derived HMPV or other heterologous viral mutant, for example, any one or combination of mutations identified in APV, HPIV, BPIV3, and/or RSV. Preferred recombinant HMPVs can incorporate a plurality of mutations thus identified. Often, these mutations are stabilized against
5 reversion in recombinant HMPV by multiple nucleotide substitutions in a codon specifying each mutation.

Mutations compiled into a "menu" as described above are introduced as desired, singly or in combination, to adjust recombinant HMPV to an appropriate level of attenuation, immunogenicity, genetic resistance to reversion from an attenuated phenotype, etc. In accordance with the foregoing
10 description, the ability to produce infectious recombinant HMPV from cDNA permits introduction of specific engineered changes within the recombinant HMPV. In particular, infectious, recombinant HMPVs can be employed for further identification of specific mutations in biologically derived, attenuated HMPV strains, for example mutations that specify *ts*, *ca*, *att* and other phenotypes. Desired mutations identified by this and other methods are introduced into recombinant HMPV
15 candidate strains. The capability of producing virus from cDNA allows for routine incorporation of these mutations, individually or in various selected combinations, into a full-length cDNA clone, where after the phenotypes of rescued recombinant viruses containing the introduced mutations can be readily determined.

By identifying and incorporating specific mutations associated with desired phenotypes, for example, a *ca* or *ts* phenotype, into infectious recombinant HMPV, additional site-specific
20 modifications at, or within close proximity to, the identified mutation are identified. Whereas most attenuating mutations produced in biologically derived HMPVs are single nucleotide changes, other "site specific" mutations can also be incorporated by recombinant techniques into a recombinant HMPV. As used herein, site-specific mutations include insertions, substitutions, deletions or
25 rearrangements of from about 1 to about 3, up to about 5-15 or more altered nucleotides (for example, altered from a wild type HMPV sequence, from a sequence of a selected mutant HMPV strain, or from a parent recombinant HMPV clone subjected to mutagenesis). Such site-specific mutations can be incorporated at, or within the region of, a selected, biologically derived point mutation. Alternatively, the mutations can be introduced in various other contexts within a recombinant HMPV
30 clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc.

Site-specific recombinant HMPV mutants typically retain a desired attenuating phenotype, but can additionally exhibit altered phenotypic characteristics unrelated to attenuation, for example, enhanced or broadened immunogenicity, and/or improved growth. Further examples of desired, site-
35 specific mutants include recombinant HMPV mutants engineered to incorporate additional, stabilizing nucleotide mutations in a codon specifying an attenuating point mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid

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changes in a parent mutant or recombinant HMPV clone, yielding a recombinant HMPV with greater genetic resistance to reversion from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream or downstream, e. g., from about 1 to about 3, about 5-10 and up to 15 nucleotides or more 5' or 3', relative to a targeted nucleotide position, for example, to construct or ablate an existing cis-acting regulatory element.

In addition to single and multiple point mutations and site-specific mutations, changes to the recombinant HMPV include deletions, insertions, substitutions or rearrangements of one or more genes or genome segments to increase, decrease, ablate or otherwise alter gene expression. Expression of one or more non-essential genes can be reduced or ablated by modifying the recombinant HMPV genome or antigenome, for example, to incorporate a mutation that alters the coding assignment of an initiation codon or mutations that introduce one or one or more stop codons. Alternatively, one or more non-essential genes or genome segments can be deleted in whole or in part to render the corresponding proteins partially or entirely non-functional or to disrupt protein expression altogether. Exemplary recombinant HMPV within these aspects of the current disclosure are provided herein that exhibit partial or complete deletions of the G, SH and/or M2-2 ORFs, including an exemplary mutant that has both the G and SH genes deleted. These and other recombinants can be engineered and selected to possess highly desirable phenotypic characteristics for development of immunogenic compositions. For example, these modifications can specify one or more desired phenotypic changes including (i) altered growth properties in cell culture, (ii) attenuation in the upper and/or lower respiratory tract of mammals, (iii) a change in viral plaque size, (iv) a change in cytopathic effect, (v) a change in immunogenicity, and (vi) a change in RNA replication and gene expression.

Thus a recombinant HMPV can incorporate one or more partial or complete gene deletions, knock out mutations, or mutations that simply reduce or increase expression of an HMPV gene. This can be achieved, for example, by introducing a frame shift mutation or termination codon within a selected coding sequence, altering translational start sites, changing the position of a gene or introducing an upstream start codon to alter its rate of expression, changing GS and/or GE transcription signals to alter phenotype, or modifying an RNA editing site (for example, growth, temperature restrictions on transcription, etc.). In several examples, recombinant HMPVs are provided in which expression of one or more genes, for example, a SH, G, or M2-2 ORF, is ablated at the translational or transcriptional level without deletion of the gene or of a segment thereof, by, for example, introducing multiple translational termination codons into a translational ORF, altering an initiation codon, or modifying an editing site. In one embodiment, expression of the M2-2 ORF is ablated by removal of both potential translational start codons, in combination with partial deletion of the M2-2 ORF. These and other forms of knock-out virus will often exhibit reduced growth rates and small plaque sizes in tissue culture. Thus, additional novel types of attenuating mutations are

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provided herein which ablate or alter expression of a viral gene. In this context, knock-out virus phenotypes produced without deletion of a gene or genome segment can be alternatively produced by deletion mutagenesis, as described, to effectively preclude correcting mutations that may restore synthesis of a target protein. Other gene knock-outs can be made using alternate designs and methods that are well known in the art (as described, for example, in (Kretschmer *et al.*, Virology 216:309-316, 1996; Radecke *et al.*, Virology 217:418-421, 1996; Kato *et al.*, EMBO J. 16:578-587, 1987; and Schneider *et al.*, Virology 277:314-322, 1996).

Nucleotide modifications that may be introduced into recombinant HMPV constructs can alter small numbers of bases (for example, from 15-30 bases, up to 35-50 bases or more), large blocks of nucleotides (for example, about 50-100, about 100-300, about 300-500, about 500-1,000 bases), or nearly complete or complete genes (for example, about 1,000-1,500 nucleotides, about 1,500-2,500 nucleotides, about 2,500-5,000, nucleotides, about 5,000-6,000 nucleotides or more) in the vector genome or antigenome or heterologous, donor gene or genome segment, depending upon the nature of the change (that is, a small number of bases may be changed to insert or ablate an immunogenic epitope or change a small genome segment, whereas large blocks of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged).

In related aspects, the current disclosure provides for supplementation of mutations adopted into a recombinant HMPV clone from biologically derived HMPV, for example, *ca* and *ts* mutations, with additional types of mutations involving the same or different genes in a further modified recombinant HMPV. Each of the HMPV genes can be selectively altered in terms of expression levels, or can be added, deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to yield a recombinant HMPV exhibiting novel characteristics. Thus, in addition to or in combination with attenuating mutations adopted from biologically derived HMPV and/or non-HMPV mutants, the current disclosure also provides a range of additional methods for attenuating or otherwise modifying the phenotype of a recombinant HMPV based on recombinant engineering of infectious HMPV clones. A variety of alterations can be produced in an isolated polynucleotide sequence encoding a targeted gene or genome segment, including a donor or recipient gene or genome segment in a recombinant HMPV genome or antigenome for incorporation into partially attenuated, infectious clones. More specifically, to achieve desired structural and phenotypic changes in recombinant HMPV, the current disclosure allows for introduction of modifications which delete, substitute, introduce, or rearrange a selected nucleotide or nucleotide sequence from a parent genome or antigenome, as well as mutations which delete, substitute, introduce or rearrange whole genes or genome segments, within a recombinant HMPV.

Thus provided are modifications in recombinant HMPV which simply alter or ablate expression of a selected gene, for example, by introducing a termination codon within a selected HMPV coding sequence or altering its translational start site or RNA editing site, changing the

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position of a HMPV gene relative to an operably linked promoter, introducing an upstream start codon to alter rates of expression, modifying (for example, by changing position, altering an existing sequence, or substituting an existing sequence with a heterologous sequence) GS and/or GE transcription signals to alter phenotype (for example, growth, temperature restrictions on transcription, etc.), and various other deletions, substitutions, additions and rearrangements that specify quantitative or qualitative changes in viral replication, transcription of selected genes, or translation of selected proteins. In this context, any HMPV gene or genome segment which is not essential for growth can be ablated or otherwise modified in a recombinant HMPV to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. In addition to coding sequences, noncoding, leader, trailer and intergenic regions can be similarly deleted, substituted or modified and their phenotypic effects readily analyzed, for example, by the use of minireplicons, and the recombinant HMPV described herein.

In addition to these changes, the order of genes in a recombinant HMPV construct can be changed, a HMPV genome promoter replaced with its antigenome counterpart or vice versa, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Other mutations for incorporation into recombinant HMPV constructs of the current disclosure include mutations directed toward cis-acting signals, which can be readily identified, for example, by mutational analysis of HMPV minigenomes. For example, insertional and deletional analysis of the leader, trailer and/or flanking sequences identifies viral promoters and transcription signals and provides a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide alternatives) of these cis-acting signals also can be employed to identify many mutations that affect RNA replication or transcription. Any of these mutations can be inserted into a chimeric HMPV antigenome or genome as described herein. Evaluation and manipulation of trans-acting proteins and cis-acting RNA sequences using the complete antigenome cDNA is assisted by the use of HMPV minigenomes as described in the above-incorporated references.

Additional mutations within recombinant HMPVs can also include replacement of the 3' end of genome with its counterpart from antigenome or vice versa, which is associated with changes in RNA replication and transcription. In one exemplary embodiment, the level of expression of specific HMPV proteins, such as the protective HN and/or F antigens, can be increased by substituting the natural sequences with ones which have been made synthetically and designed to be consistent with efficient translation. In this context, it has been shown that codon usage can be a major factor in the level of translation of mammalian viral proteins (Haas *et al.*, Current Biol. 6:315-324, 1996). Optimization by recombinant methods of the codon usage of the mRNAs encoding one or more

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immunogenic glycoproteins of recombinant HMPV will provide improved expression for these genes.

In another exemplary embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position relative to the AUG start site) of a selected HMPV gene or donor gene incorporated in an HMPV vector is modified, alone or in combination with introduction of an upstream start codon, to modulate gene expression by specifying up- or down-regulation of translation. Alternatively, or in combination with other recombinant modifications disclosed herein, gene expression of a recombinant HMPV can be modulated by altering a transcriptional GS or GE signal of any selected genes of the virus. In alternative embodiments, levels of gene expression in a recombinant HMPV candidate are modified at the level of transcription. In one aspect, the position of a selected gene in the HMPV gene map can be changed to a more promoter-proximal or promoter-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be achieved yielding reductions or increases of gene expression from two-fold, more typically four-fold, up to ten-fold or more compared to wild type levels often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes. These and other transpositioning changes yield novel recombinant HMPVs having attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, or having other desirable properties such as increased antigen expression.

In the latter context, any one or combination of antigenic glycoproteins, prospectively including F, G, and SH, or antigenic determinants thereof, can be produced at elevated or decreased levels (or with otherwise enhanced immunogenic activity) by, for example, changing the promoter-relative position of the subject gene or genome segment. For example, the wild type gene order of the SH, G and F ORFs of HMPV are located at gene positions 6, 7 and 4, respectively. Recombinantly altering the promoter proximity of one or more of these genes will modulated its expression, and/or expression of normally "upstream" and "downstream" genes. The change in gene expression due to the relocation of genes or to the addition of a second copy can have other desirable effects, such as increased or decreased growth *in vitro* or *in vivo*. In exemplary embodiments, placement of putative protective antigen genes such as SH, G, and F more proximal to the promoter, either singly, as a pair, or as a triplet, will result in more efficient expression. As described herein, exemplary embodiments of "promoter-shifted" HMPV are provided wherein the position of the SH-G gene pair was altered, for example by moving the pair from its wild type gene order position following the M2 gene to a position preceding the F gene. This resulted in a gene order in that region of the genome that mimics that of RSV. In another exemplary embodiment, the SH-G pair was moved to be upstream of the F gene, and a second copy of the pair was inserted, resulting in multiple copies of the SH and G prospective antigens. In yet additional embodiments, the prospective antigens comprising one or more of the G and F proteins are modified in their expression by

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promoter-relative shifting of these genes (having wild type gene order positions at the 7th and 4th gene positions, respectively in the HMPV genome map) to more promoter-proximal positions.

In other embodiments, recombinant HMPVs useful in immunogenic compositions can be conveniently modified to accommodate antigenic drift in circulating virus. In this context, the
5 Examples below provide the complete sequence for an HMPV genome, and also provide a detailed comparison of complete genome sequences for HMPV isolates representing two distinct antigenic subgroups of HMPV, that are generally comparable in sequence divergence to the antigenic subgroups of HRSV. Identification and characterization of these distinct antigenic subgroups, including detailed mapping of their prospective antigenic glycoprotein gene structure, provides for
10 construction of novel recombinant HMPVs for use in immunogenic compositions to elicit an immune response against one or more HMPV strains, including in bivalent immunogenic composition to immunize against multiple HMPV strains and/or accommodate antigenic drift. Typically the recombinant HMPV will have a modification in one or more of the SH, G, and/or F glycoproteins. For example, an entire SH, G, and/or F gene, or a genome segment encoding a particular antigenic
15 determinant (for example, immunogenic region or epitopes) thereof, from one HMPV strain or group can be incorporated into a recombinant HMPV genome or antigenome cDNA by replacement of a corresponding region in a recipient clone of a different HMPV strain or group, or by adding one or more copies of the gene, such that multiple antigenic forms are represented in the recombinant virus. Progeny virus produced from the modified recombinant HMPV can then be used in immunization
20 protocols against multiple, and emerging, HMPV strains.

In certain aspects of the current disclosure, replacement of a HMPV coding sequence or non-coding sequence (for example, a promoter, GE, GS, intergenic or other cis-acting element) with a heterologous (for example, non-HMPV) counterpart yields chimeric HMPV having a variety of possible attenuating and other phenotypic effects. For example, host range and other desired effects
25 can be engineered by importing an APV or other non-HMPV virus (for example, RSV, SV5, SV41, NDV, PIV) protein, protein domain, gene or genome segment into a recombinant HMPV “background” genome or antigenome, wherein the heterologous or “donor” gene or genome segment does not function efficiently in a human cell, for example, from incompatibility of the heterologous sequence or protein with a biologically interactive HMPV sequence or protein (that is, a sequence or
30 protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.) or, more typically in a host range restriction, with a cellular protein or some other aspect of the cellular milieu which is different between the permissive and less permissive host. In exemplary embodiments, APV, pneumovirus of mice, or RSV sequences are selected for introduction into HMPV based on aspects of HMPV structure and function described herein.

35 Methods are provided herein for attenuating recombinant HMPV candidates based on the construction of chimeras between HMPV and a non-human pneumovirus or other negative stranded RNA virus, for example APV, RSV, HPIV, MPIV1 (Sendai virus), BPIV3, SV5, SV41, and/or NDV

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(for example, as disclosed in U.S. Patent Application Serial No. 09/586,479, filed June 1, 2000 by Schmidt *et al.* (corresponding to PCT Publication WO 01/04320); Schmidt *et al.*, J. Virol. 74:8922-9, 2000). In exemplary embodiments, the recombinant HMPV genome or antigenome is combined with a heterologous gene or genome segment, such as an N, P, M, or L, ORF derived from a non-HMPV paramyxovirus.

Paramyxoviruses are enveloped RNA viruses that include two subfamilies (*Paramyxovirinae* and *Pneumovirinae*). Subfamily *Paramyxovirinae* has five different genera, including *Respirovirus* (containing parainfluenza viruses 1 and 3), *Rubulavirus* (containing parainfluenza viruses 2 and 4 and mumps), and *Morbillivirus* (containing measles and canine distemper viruses). Subfamily *Pneumovirinae* has two genera, namely *Pneumovirus* (containing RSV) and *Metapneumovirus* (containing HMPV). Paramyxoviruses encoded 6-11 proteins. Proteins common to all paramyxoviruses include the nucleocapsid N protein that associates tightly with the RNA genome to form the nucleocapsid, the phosphoprotein P and large L polymerase protein that also associate with the nucleocapsid, the matrix M protein that associates with the inner face of the viral envelope, the transmembrane fusion F glycoprotein that mediates penetration, and the attachment protein that, depending on the virus, is called glycoprotein G, or the hemagglutinin HA or the hemagglutinin-neuraminidase HN. Other proteins are found in some viruses but not others, such as the nonstructural NS1 and NS2 proteins of RSV, the M2-1 and M2-2 proteins found in RSV and, as disclosed here, in HMPV, the SH protein that is found in mumps, simian virus 5, RSV and HMPV, and the C, D and V proteins found in certain parainfluenza viruses. The sequences of non-HMPV HN, M, N, P, L, F, G, and SH proteins, and nucleic acids encoding these proteins are known in the art (see, Lamb, RA and Kolakofsky, D. pp 1305-1340 in Fields Virology, 4th addition, Knipe and Howley (eds) Lippincott Williams and Wilkins, Philadelphia, 2001, Chanock, Murphy and Collins, pp 1341-1379, *ibid*, Collins, Chanock and Murphy, pp 1443-1485, *ibid*.).

Chimeric HMPV are therefore provided herein that include a partial or complete “background” HMPV genome or antigenome derived from or patterned after HMPV combined with one or more heterologous genes or genome segments of a non- HMPV virus to form the chimeric HMPV genome or antigenome. In one embodiment, chimeric HMPV of this type incorporate a partial or complete HMPV background genome or antigenome combined with one or more heterologous genes or genome segments. The partial or complete background genome or antigenome typically acts as a recipient backbone into which the heterologous genes or genome segments of the counterpart, non- HMPV virus are incorporated. Heterologous genes or genome segments from the counterpart virus represent “donor” genes or polynucleotides that are combined with, or substituted within, the background genome or antigenome to yield a chimeric HMPV that exhibits novel phenotypic characteristics compared to one or both of the contributing viruses. For example, addition or substitution of heterologous genes or genome segments within a selected recipient HMPV strain may result in an increase or decrease in attenuation, growth changes, altered immunogenicity,

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or other desired phenotypic changes as compared with a corresponding phenotypes of the unmodified recipient and/or donor (see, for example, U.S. Patent Application Serial No. 09/586,479, filed June 1, 2000 by Schmidt *et al.*; Schmidt *et al.*, J. Virol. 74:8922-9, 2000).

5 Genes and genome segments that can be selected for use as heterologous substitutions or additions within chimeric HMPV include genes or genome segments encoding a HMPV N, P, M, F, M2, SH, G and/or L proteins or portions thereof. In addition, genes and genome segments encoding proteins found in other viruses, (for example, an SH protein as found in mumps, RSV, and SV5 viruses), may be incorporated within additional chimeric HMPV recombinants of the current disclosure. Regulatory regions from heterologous viruses, for example the extragenic 3' leader or 5' trailer regions, and GS, GE, intergenic regions, or 3' or 5' non-coding regions, are also useful as substitutions or additions within recombinant HMPV. In exemplary aspects, chimeric HMPV bearing one or more non-HMPV genes or genome segments exhibit a high degree of host range restriction, for example, in the respiratory tract of mammalian models of human HMPV infection such as hamsters and non-human primates. In more detailed embodiments HMPV is attenuated by the addition or substitution of one or more APV or RSV genes or genome segments selected from N, P, M, F, M2, SH, G and/or L genes and genome segments to a partial or complete HMPV background genome or antigenome.

15 In one embodiment, the degree of host range restriction exhibited by chimeric HMPV for use within immunogenic compositions of the current disclosure is comparable to the degree of host range restriction exhibited by the respective non-HMPV "donor" strain. For example, the restriction should have a true host range phenotype, that is, it should be specific to the host in question and should not restrict replication *in vitro* in a suitable cell line. In addition, chimeric HMPV bearing one or more heterologous genes or genome segments elicit a desired immunogenic response in hosts susceptible to HMPV infection. Thus, the current disclosure provides a new basis for attenuating a live HMPV virus vector for developing immunogenic compositions against HMPV and other pathogens based on host range effects.

25 In combination with the host range phenotypic effects provided in the chimeric HMPV, it is often desirable to adjust the attenuation phenotype by introducing additional mutations that increase or decrease attenuation of the chimeric virus. Thus, in additional embodiments, attenuated, chimeric HMPV are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations specifying an attenuating phenotype in the resultant virus or subviral particle. These can include mutations generated *de novo* and tested for attenuating effects according to a rational design mutagenesis strategy. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant HMPV or non-HMPVs and thereafter incorporated into a chimeric HMPV of the current disclosure. Exemplary mutations specify lesions in RNA regulatory sequences or in encoded proteins.

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In certain chimeric HMPV, attenuation marked by replication in the lower and/or upper respiratory tract in an accepted animal model that is reasonably correlated with HMPV replication and immunogenic activity in humans (for example, hamsters, rhesus monkeys or chimpanzees), is reduced by at least about two-fold, more often about 5-fold, 10-fold, or 20-fold, and preferably 50-
5 100-fold and up to 1,000-fold or greater overall (for example, as measured between 3-8 days following infection) compared to growth of the corresponding wild type or mutant parental strains.

Within the methods disclosed herein, additional genes or genome segments can be inserted into or proximate to a recombinant or chimeric HMPV genome or antigenome. For example, various supernumerary heterologous genes or genome segments can be inserted at any of a variety of sites
10 within the recombinant genome or antigenome, for example at a position 3' to N, between the N/P, P/M, M/F, F/M2, M2/SH, SH/G, and/or G/L genes, or at another non-coding region of the HMPV vector genome or antigenome (see FIG. 1). The inserted genes can be under common control with recipient genes, or can be under the control of an independent set of transcription signals. Genes of interest in this context include genes encoding cytokines, for example, an interleukin (IL-2 through
15 IL-18, for example, interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 18 (IL-18), tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), or granulocyte-macrophage colony stimulating factor (GM-CSF) (see, for example, U.S. Application No. 09/614,285, filed July 12, 2000 and priority U.S. Provisional Application Serial No. 60/143,425 filed July 13, 1999). Coexpression of these additional proteins provides the ability to modify and
20 improve immune responses against recombinant HMPV quantitatively and/or qualitatively.

In additional embodiments, insertion of heterologous nucleotide sequences into recombinant HMPV candidates are employed separately to modulate the level of attenuation of candidate recombinants, for example, for the upper respiratory tract. Thus, it is possible to insert nucleotide sequences into a recombinant HMPV that both direct the expression of a foreign protein and that
25 attenuate the virus in an animal host, or to use nucleotide insertions separately to attenuate candidate viruses. To define some of the rules that govern the effect of gene insertion on attenuation, gene units of varying lengths are inserted into a wild type HMPV backbone and the effects of gene unit length on attenuation examined. Novel gene unit insertions are contemplated in this regard that do not contain a significant ORF, permitting identification of the effect of gene unit length independently of
30 an effect of the expressed protein of that gene. These heterologous sequences may be inserted as an extra gene unit of various sizes, for example, from about 150 or more nts in length up to 3,000 nts or more in length. Gene unit (GU) insertions of about 1,000 or 2,000 nts in length will often substantially attenuate rHMPV candidates for the upper respiratory tract of mammalian subjects. In addition, gene unit insertions can have the dual effect of both attenuating a candidate virus and
35 inducing an immunogenic response against a second virus. Alternately, gene extensions in the 3'-noncoding region (NCR) of a HMPV gene, which cannot express additional proteins, can also be attenuating in and of themselves. Within these methods of the current disclosure, gene insertion

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length is a determinant of attenuation (see, for example, U.S. Patent Application No. 10/302,547, filed November 21, 2002 and priority U.S. Provisional Application No. 60/331,961, filed November 21, 2001; and U.S. Provisional Application No. 60/412,053, filed September 18, 2002).

5 Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or genome segments within rHMPV yield highly stable candidates, which are particularly important in the case of immunosuppressed individuals. Many of these changes will result in attenuation of resultant strains, whereas others will specify different types of desired phenotypic changes. For example, accessory (that is, not essential for *in vitro* growth) genes are excellent candidates to encode proteins that specifically interfere with host immunity (see, for example, Kato *et* 10 *al.*, EMBO. J. 16:578-87, 1997). Ablation of such genes in candidate viruses can reduce virulence and pathogenesis and/or improve immunogenicity.

In more detailed embodiments, chimeric HMPVs are constructed using a HMPV “vector” genome or antigenome that is recombinantly modified to incorporate one or more antigenic determinants of a heterologous pathogen. The vector genome or antigenome includes a partial or 15 complete HMPV genome or antigenome, which may itself incorporate nucleotide modifications such as attenuating mutations. The vector genome or antigenome is modified to form a chimeric structure through incorporation of a heterologous gene or genome segment. More specifically, chimeric HMPVs of the current disclosure are constructed through a cDNA-based virus recovery system that yields recombinant viruses that incorporate a partial or complete vector or “background” HMPV 20 genome or antigenome combined with one or more “donor” nucleotide sequences encoding the heterologous antigenic determinants. In exemplary embodiments a HMPV vector genome or antigenome is modified to incorporate one or more genes or genome segments that encode antigenic determinants of one or more heterologous negative stranded RNA viruses (for example, another HMPV, RSV, PIV, or measles virus). Thus constructed, chimeric HMPVs can elicit, for example, an 25 immune response against a specific HMPV or against a non-HMPV pathogen. Alternatively, compositions and methods are provided employing a HMPV-based chimeric virus to elicit a polyspecific immune response against multiple HPIVs, against one or more HMPVs and a non-PIV pathogen such as RSV, PIV, or measles virus.

A chimeric HMPV in this context can incorporate a partial or complete human HMPV 30 incorporating one or more heterologous polynucleotides encoding one or more antigenic determinants of the heterologous pathogen. These heterologous polynucleotides can be added to or substituted within the HMPV vector genome or antigenome to yield the chimeric HMPV recombinant. The chimeric HMPV virus thus acquires the ability to elicit an immune response in a selected host against the heterologous pathogen. In addition, the chimeric virus can exhibit other novel phenotypic 35 characteristics compared to one or both of the vector HMPV and heterologous pathogens. In addition to providing novel immunogenic characteristics, the addition or substitution of heterologous genes or genome segments within the vector HMPV strain may confer an increase or decrease in attenuation,

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growth changes, or other desired phenotypic changes as compared with the corresponding phenotypes of the unmodified vector and donor viruses.

Heterologous genes or genome segments of a different HMPV or non-HMPV virus can be added as a supernumerary genomic element to a partial or complete genome or antigenome of a rHMPV. Alternatively, one or more heterologous genes or genome segments may be substituted into the rHMPV at a position corresponding to a wild type gene order position of a counterpart genes or genome segments that is deleted within the HMPV vector genome or antigenome. In yet additional embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild type gene order position of the counterpart gene or genome segment within the vector genome or antigenome to enhance or reduce, respectively, expression of the heterologous gene or genome segment. Additional methods and compositions that are useful for engineering chimeric HMPV employ certain known techniques, for example, as disclosed for PIV viruses (Durbin *et al.*, Virology 235:323-332, 1997; Skiadopoulos *et al.*, J. Virol. 72:1762-1768, 1998; Tao *et al.*, J Virol 72:2955-2961, 1998; Skiadopoulos *et al.*, J. Virol. 73:1374-1381, 1999; Skiadopoulos *et al.*, Vaccine 18:503-510, 1999; Tao *et al.*, Vaccine 17:1100-1108, 1999; Tao *et al.*, Vaccine 18:1359-1366, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997).

Chimeric HMPV can also be constructed that express a chimeric protein, for example an immunogenic glycoprotein having a cytoplasmic tail and/or transmembrane domain specific to a HMPV vector fused to a heterologous ectodomain (the ectodomain being that part of a transmembrane surface protein that extends into the luminal or extracellular space) of a different HMPV or non-HMPV pathogen to provide a fusion protein that elicits an immune response against the heterologous pathogen. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a RSV F, SH, M2, or G glycoprotein can be joined with a genome segment encoding the corresponding HMPV glycoprotein cytoplasmic and transmembrane domains to form a chimeric glycoprotein that elicits an immune response against RSV.

Briefly, HMPV expressing a chimeric glycoprotein includes a major nucleocapsid protein, a nucleocapsid phosphoprotein, a large polymerase protein, and a HMPV vector genome or antigenome that is modified to encode a chimeric glycoprotein. The chimeric glycoprotein incorporates one or more heterologous antigenic domains, fragments, or epitopes of a second, antigenically distinct HMPV or non-HMPV pathogen. In one embodiment, this is achieved by substitution within the HMPV vector genome or antigenome of one or more heterologous genome segments of the second virus that encodes one or more antigenic domains, fragments, or epitopes, whereby the recombinant genome or antigenome encodes the chimeric glycoprotein that is antigenically distinct from the

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parent, vector virus. In several examples, the heterologous genome segment or segments can encode a glycoprotein ectodomain or immunogenic portion or epitope thereof. The heterologous genome segment can also include other portions of the heterologous or "donor" glycoprotein, for example both an ectodomain and transmembrane region that are substituted for counterpart glycoprotein ecto- and transmembrane domains in the vector genome or antigenome.

As used herein, the term "gene" generally refers to a portion of a subject genome, for example, a HMPV genome, encoding an mRNA and typically begins at the upstream end with a GS signal and ends at the downstream end with the GE signal. The term gene is also interchangeable with the term "translational open reading frame," or "ORF," particularly in the case where a protein, such as the HPIV1 or HPIV3 C protein, is expressed from an additional ORF rather than from a unique mRNA. The viral genome of all mononegaviruses also contains extragenic leader and trailer regions, possessing part of the promoters required for viral replication and transcription, as well as non-coding and intergenic regions. Transcription initiates at the 3' end and proceeds by a sequential stop-start mechanism that is guided by short conserved motifs found at the gene boundaries. The upstream end of each gene contains a GS signal, that directs initiation of its respective mRNA. The downstream terminus of each gene contains a GE motif that directs polyadenylation and termination. The current disclosure provides operative identification of transcription signals and insertion sites, and provides the identification of the GS, GE and potential insertion sites within the genome.

To construct chimeric HMPVs, one or more HMPV genes or genome segments can be deleted, inserted or substituted in whole or in part. This means that partial or complete deletions, insertions and substitutions may include ORFs and/or cis-acting regulatory sequences of any one or more of the HMPV genes or genome segments. By "genome segment" is meant any length of continuous nucleotides from the HMPV genome, which might be part of an ORF, a gene, or an extragenic region, or a combination thereof. In several examples, at least about 10, at least about 20, at least about 30, at least about 50, or at least about 100 continuous nucleotides are deleted, inserted or substituted. When a subject genome segment encodes an antigenic determinant, the genome segment encodes at least one immunogenic epitope capable of eliciting a humoral or cell mediated immune response in a mammalian host. The genome segment can also encode an immunogenic fragment or protein domain. For example, the donor genome segment can encode multiple immunogenic domains or epitopes, including recombinantly synthesized sequences that comprise multiple, repeating or different, immunogenic domains or epitopes.

Chimeric HMPV can be engineered to express one or more major antigenic determinants of a wide range of non-HMPV pathogens. The methods disclosed herein are generally adaptable for incorporation of antigenic determinants from, for example, subgroup A and subgroup B RSVs, HMPV, measles virus, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses, among other pathogens.

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Pathogens that can be targeted for development of immunogenic compositions include viral and bacterial pathogens, as well as protozoans and multicellular pathogens. Useful antigenic determinants from many important human pathogens in this context are known or readily identified for incorporation within chimeric HMPV. Thus, major antigens have been identified for the foregoing exemplary pathogens, including the measles virus HA and F proteins; the F, G, SH and M2 proteins of RSV, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, flavivirus E and NS1 proteins, and alphavirus E protein. These major antigens, as well as other antigens known in the art for the enumerated pathogens and others, are well characterized to the extent that many of their antigenic determinants, including the full length proteins and their constituent antigenic domains, fragments and epitopes, are identified, mapped and characterized for their respective immunogenic activities.

Among the numerous, exemplary mapping studies that identify and characterize major antigens of diverse pathogens for use in the methods a chimeric HMPV disclosed herein are epitope mapping studies directed, for example, to immunogenic glycoproteins of PIV. Exemplifying the subject methods and tools, van Wyke Coelingh *et al.* (J. Virol. 63:375-382, 1989) described twenty-six monoclonal antibodies (MAbs) (14 neutralizing and 12 nonneutralizing) that were used to examine the antigenic structure, biological properties, and natural variation of the fusion (F) glycoprotein of HPIV3. Analysis of laboratory-selected antigenic variants and of PIV3 clinical isolates indicated that the panel of MAbs recognizes at least 20 epitopes, 14 of which participate in neutralization. Competitive binding assays confirmed that the 14 neutralization epitopes are organized into three nonoverlapping principal antigenic regions (A, B, and C) and one bridge site (AB), and that the 6 nonneutralization epitopes form four sites (D, E, F, and G). Most of the neutralizing MAbs were involved in nonreciprocal competitive binding reactions, suggesting that they induce conformational changes in other neutralization epitopes. These and related methods will serve to readily determine candidate antigenic determinants among heterologous viruses for expression by HMPV vectors of the current disclosure.

Other antigenic determinants for use within the current disclosure have been identified and characterized for RSV. For example, Beeler *et al.*, J. Virol. 63:2941-2950, 1989, employed eighteen neutralizing monoclonal antibodies (MAbs) specific for the fusion glycoprotein of the A2 strain of RSV to construct a detailed topological and operational map of epitopes involved in RSV neutralization and fusion. Competitive binding assays identified three nonoverlapping antigenic regions (A, B, and C) and one bridge site (AB). Thirteen MAb-resistant mutants (MARMs) were selected, and the neutralization patterns of the MAbs with either MARMs or RSV clinical strains identified a minimum of 16 epitopes. MARMs selected with antibodies to six of the site A and AB epitopes displayed a small-plaque phenotype, which is consistent with an alteration in a biologically

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active region of the F molecule. Analysis of MARMs also indicated that these neutralization epitopes occupy topographically distinct but conformationally interdependent regions with unique biological and immunological properties. Antigenic variation in F epitopes was then examined by using 23 clinical isolates (18 subgroup A and 5 subgroup B) in cross-neutralization assays with the 18 anti-F

5 MAbs. This analysis identified constant, variable, and hypervariable regions on the molecule and indicated that antigenic variation in the neutralization epitopes of the RSV F glycoprotein is the result of a noncumulative genetic heterogeneity. Of the 16 epitopes, 8 were conserved on all or all but 1 of 23 subgroup A or subgroup B clinical isolates. These antigenic determinants, including the full

10 length proteins and their constituent antigenic domains, fragments and epitopes, all represent useful candidates for integration within chimeric HMPV of the current disclosure to elicit novel immune responses as described above. (See also, Anderson *et al.*, J. Infect. Dis. 151:626-633, 1985; Coelingh *et al.*, J. Virol. 63:375-382, 1989; Fenner *et al.*, Scand. J. Immunol. 24:335-340, 1986; Fernie *et al.*, Proc. Soc. Exp. Biol. Med. 171:266-271, 1982; Sato *et al.*, J. Gen. Virol. 66:1397-1409, 1985; Walsh *et al.*, J. Gen. Virol. 67:505-513, 1986, and Olmsted *et al.*, J. Virol. 63:411-420, 1989).

15 To express antigenic determinants of heterologous HMPVs and non-HMPV pathogens, numerous methods and constructs are provided herein. In certain detailed embodiments, a transcription unit comprising an ORF of a gene encoding an antigenic protein (for example, an RSV F or G gene) is added to a HMPV vector genome or antigenome at various positions, yielding exemplary chimeric "vector" candidates. In exemplary embodiments, chimeric HMPVs are

20 engineered that incorporate heterologous nucleotide sequences encoding protective antigens from RSV to produce infectious, attenuated candidates (see U.S. Patent Application No. 08/720,132, filed September 27, 1996, corresponding to International Publication WO 97/12032 published April 04/03/97, and U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent

25 Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent Application No. 08/892,403, filed July 15, 1997 corresponding to International Publication No. WO 98/02530 published on January 22, 1998; U.S. Patent Application No. 09/291,894, filed on April 13, 1999 corresponding to International Publication No. WO 00/61611 published October 19, 2000, and priority U.S. Provisional Patent Application Serial No. 60/129,006,

30 filed on April 13, 1999; U.S. Patent Application No. 09/602,212, filed June 23, 2000 and corresponding International Publication No. WO 01/04335 published on January 18, 2001, and priority U.S. Provisional Patent Application Nos. 60/129,006, filed April 13, 1999, 60/143,097, filed July 9, 1999, and 60/143,132, filed July 9, 1999; International Publication No. WO 00/61737 published on October 19, 2000; Collins *et al.*, Proc Nat. Acad. Sci. U.S.A. 92:11563-11567, 1995;

35 Bukreyev *et al.*, J. Virol. 70:6634-41, 1996, Juhasz *et al.*, J. Virol. 71:5814-5819, 1997; Durbin *et al.*, Virology 235:323-332, 1997; He *et al.* Virology 237:249-260, 1997; Baron *et al.* J. Virol. 71:1265-1271, 1997; Whitehead *et al.*, Virology 247:232-9, 1998a; Whitehead *et al.*, J. Virol. 72:4467-4471,

1998b; Jin *et al.* Virology 251:206-214, 1998; and Whitehead *et al.*, J. Virol. 73:3438-3442, 1999, and Bukreyev *et al.*, Proc. Nat. Acad. Sci. U.S.A. 96:2367-72, 1999). Other reports and discussion incorporated or set forth herein identify and characterize RSV antigenic determinants that are useful within the current disclosure.

5 HMPV chimeras incorporating one or more RSV antigenic determinants, can include a HMPV vector genome or antigenome combined with a heterologous gene or genome segment encoding an antigenic RSV glycoprotein, protein domain (for example, a glycoprotein ectodomain) or one or more immunogenic epitopes. In one embodiment, one or more genes or genome segments from RSV SH, M2, F, and/or G genes is/are combined with the HMPV vector genome or antigenome
10 to form the chimeric HMPV candidate. Certain of these constructs will express chimeric proteins, for example fusion proteins having a cytoplasmic tail and/or transmembrane domain of HMPV fused to an ectodomain of a corresponding RSV glycoprotein to yield a novel attenuated virus that optionally elicits a multivalent immune response against both HMPV and RSV.

In certain embodiments, it is useful to administer immunogenic compositions comprising a
15 rHMPV in a predetermined schedule with one or more additional immunogenic components, for example a second immunogenic composition against RSV or PIV administered before or after the anti-HMPV composition. RSV and HPIV3 cause significant illness within the first four months of life whereas most of the illness caused by HPIV1 and HPIV2 occur after six months of age (Chanock
20 *et al.*, in Parainfluenza Viruses, Knipe *et al.* (Eds.), pp. 1341-1379, Lippincott Williams & Wilkins, Philadelphia, 2001; Collins *et al.*, In Fields Virology, Vol. 1, pp. 1205-1243, Lippincott-Raven Publishers, Philadelphia, 1996; Reed *et al.*, J. Infect. Dis. 175:807-13, 1997). Accordingly, certain sequential immunization protocols involve administration of a immunogenic composition as described herein that elicits an immune response against HMPV before, simultaneous with (for
25 example, as a combined immunogenic composition), or subsequent to, administration of a second immunogenic composition directed toward another virus. In one embodiment, an immunogenic composition that elicits an immune response against HMPV, or against HMPV and RSV is administered one, two or more times early in life, with the first dose administered at or before one month of age, followed by an immunogenic composition against HPIV1 and/or HPIV2 at about four and six months of age. Alternatively, it might be advantageous to administer immunogenic
30 components against HMPV, RSV, and one or more PIVs at the same time, perhaps as a multi-component immunogenic composition.

Thus, combinatorial immunogenic compositions and coordinate immunization protocols are provided herein for multiple pathogenic agents, including HMPV, RSV, and one or more PIVs. In
35 exemplary embodiments, these methods and formulations are temporally selected to target early immunization against HMPV, RSV and/or PIV3. One exemplary immunization sequence employs one or more live attenuated immunogenic compositions against HMPV, RSV and/or PIV3 as early as one month of age (for example, at one and two months of age) followed by mono- or bivalent PIV1

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and/or PIV2 immunogenic composition at four and six months of age. The methods disclosed herein can be used to administer multiple immunogenic compositions, including one or more chimeric HMPV candidates, coordinately, for example, simultaneously in a mixture or separately in a defined temporal sequence (for example, in a daily or weekly sequence), wherein each virus preferably expresses, for example, a different heterologous protective antigen. Such a coordinate/sequential immunization strategy, which is able to induce secondary antibody responses to multiple viral respiratory pathogens, provides a highly powerful and extremely flexible immunization regimen that is driven by the need to immunize against multiple pathogens in early infancy.

As noted above, the current disclosure permits a wide range of alterations to be recombinantly produced within the HMPV genome or antigenome, yielding defined mutations that specify desired phenotypic changes. Defined mutations can be introduced by a variety of conventional techniques (for example, site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA constructs provide for better ease of manipulation than large cDNA constructs, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Stratagene (La Jolla, CA), or by the PCR employing either an oligonucleotide primer or a template which contains the mutations of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and can be routinely adapted for use in producing the mutations of interest in a HMPV antigenome or genome cDNA of the current disclosure.

Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid *in vitro* mutagenesis kit available from Bio-Rad Laboratories. In brief, cDNA encoding a HMPV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is then amplified.

Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome segments can correspond to structural and/or functional domains, for example, cytoplasmic, transmembrane or ectodomains of proteins, active sites such as sites that mediate binding or other biochemical interactions with different proteins, epitopic sites, for example, sites that stimulate

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antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of genome segments encoding small functional domains of proteins, for example, epitopic sites, to about 50, about 75, about 100, about 200-500, and about 500-1,500 or more nucleotides.

5 The ability to introduce defined mutations into infectious recombinant HMPV has many applications, including the manipulation of HMPV pathogenic and immunogenic mechanisms. For example, the functions of HMPV proteins, including the N, P, M, F, M2, SH, G and/or L proteins can be manipulated by introducing mutations which ablate or reduce the level of protein expression, or which yield mutant protein. Various genome RNA structural features, such as promoters, intergenic
10 regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the current disclosure. The effects of trans-acting proteins and cis-acting RNA sequences can be readily determined, for example, using a complete antigenome cDNA in parallel assays employing HMPV minigenomes (Dimock *et al.*, J. Virol. 67:2772-8, 1993 in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to
15 be recovered in replication-independent infectious virus.

Certain substitutions, insertions, deletions or rearrangements of genes or genome segments within recombinant HMPV (for example, substitutions of a genome segment encoding a selected protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.)
20 are made in structural or functional relation to an existing, "counterpart" gene or genome segment from the same or different HMPV or other source. Such modifications yield novel recombinants having desired phenotypic changes compared to wild type or parental HMPV or other viral strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one HMPV, or of a non-HMPV virus such as PIV or RSV, fused to
25 an ectodomain of a rHMPV of the current disclosure. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

As used herein, "counterpart" genes, genome segments, proteins or protein regions, are typically from heterologous sources (for example, from different HMPV genes, or representing the same (that is, homologous or allelic) gene or genome segment in different HMPV types or strains).
30 Typical counterparts selected in this context share gross structural features, for example, each counterpart can encode a comparable protein or protein structural domain, such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc. Counterpart domains and their encoding genome segments embrace an assemblage of species having a range of size and sequence variations defined by a common biological activity among the domain or
35 genome segment variants.

Counterpart genes and genome segments, as well as other polynucleotides disclosed herein for producing recombinant HMPV, often share substantial sequence identity with a selected

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polynucleotide “reference sequence,” for example, with another selected counterpart sequence. As used herein, a “reference sequence” is a defined sequence used as a basis for sequence comparison, for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) include a sequence (that is, a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) can further include a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a “comparison window” to identify and compare local regions of sequence similarity. A “comparison window”, as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (that is, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, (Adv. Appl. Math. 2:482, 1981), by the homology alignment algorithm of Needleman & Wunsch, (J. Mol. Biol. 48:443, 1970), by the search for similarity method of Pearson & Lipman, (Proc. Natl. Acad. Sci. U.S.A. 85:2444, 1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (that is, resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term “sequence identity” means that two polynucleotide sequences are identical (that is, on a nucleotide-by-nucleotide basis) over the window of comparison. The term “percentage of sequence identity” is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (for example, A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (that is, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms “substantial identity” as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of

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the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

In addition to these polynucleotide sequence relationships, proteins and protein regions encoded by recombinant HMPV are also typically selected to have conservative relationships, that is, to have substantial sequence identity or sequence similarity, with selected reference polypeptides. As applied to polypeptides, the term "sequence identity" means peptides share identical amino acids at corresponding positions. The term "sequence similarity" means peptides have identical or similar amino acids (that is, conservative substitutions) at corresponding positions. The term "substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, such as at least 90 percent sequence identity, at least 95 percent sequence identity or more (for example, 97, 98, or 99 percent sequence identity). The term "substantial similarity" means that two peptide sequences share corresponding percentages of sequence similarity. Preferably, residue positions that are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Exemplary conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Abbreviations for the twenty naturally occurring amino acids used herein follow conventional usage (Immunology - A Synthesis, 2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer Associates, Sunderland, MA, 1991). Stereoisomers (for example, D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α , α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the current disclosure. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine, and other similar amino acids and imino acids (for example, 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

To select candidate viruses, the criteria of viability, attenuation and immunogenicity are determined according to well-known methods. Viruses that will be most desired in immunogenic compositions must maintain viability, have a stable attenuation phenotype, exhibit replication in an immunized host (albeit at lower levels), and effectively elicit production of an immune response in a recipient sufficient to elicit a desired immune response. The recombinant HMPVs are not only viable

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and appropriately attenuated, they are more stable genetically *in vivo*--retaining the ability to stimulate an immune response and in some instances to expand the immune response elicited by multiple modifications, for example, induce an immune response against different viral strains or subgroups, or to stimulate a response mediated by a different immunologic basis, for example, secretory versus serum immunoglobulins, cellular immunity, and the like.

Recombinant HMPVs can be tested in various well-known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity. In *in vitro* assays, the modified virus (for example, a multiply attenuated, biologically derived or recombinant HMPV) is tested, for example, for temperature sensitivity of virus replication, that is, its phenotype, and for the small plaque or other desired phenotype. Modified viruses are further tested in animal models of HMPV infection. A variety of animal models have been described and are summarized in various references incorporated herein. HMPV model systems, including rodents and non-human primates, for evaluating attenuation and immunogenic activity of HMPV candidates are widely accepted in the art, and the data obtained there from correlate well with HMPV infection, attenuation and immunogenicity in humans.

In accordance with the foregoing description, the present disclosure also provides isolated, infectious recombinant HMPV for use in immunogenic compositions. The attenuated virus which is a component of an immunogenic composition is in an isolated and typically purified form. By "isolated" is meant to refer to HMPV which is in other than a native environment of a wild type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial medium where it can be propagated and characterized in a controlled setting. For example, attenuated HMPV can be produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

For use in immunogenic compositions, recombinant HMPV produced according to the current disclosure can be used directly in formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, for example, saline or comprising SPG, Mg^{++} and HEPES, with or without adjuvant, as further described herein.

HMPV-based immunogenic compositions contain as an active ingredient an immunogenically effective amount of a recombinant HMPV produced as described herein. The modified virus can be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, for example, water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and

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the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton IN) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known in the art.

Upon immunization with a recombinant HMPV composition, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the immunogenic composition by producing antibodies specific for HMPV proteins, for example, F and G glycoproteins. As a result of the immunization with an immunogenically effective amount of a recombinant HMPV produced as described herein, the host becomes at least partially or completely immune to infection by the targeted HMPV or non- HMPV pathogen, or resistant to developing moderate or severe infection there from, particularly of the lower respiratory tract.

The host to which the immunogenic compositions are administered can be any mammal which is susceptible to infection by HMPV or a selected non- HMPV pathogen and which host is capable of generating an immune response to the antigens of the immunizing strain. Accordingly, methods are provided for creating immunogenic compositions for a variety of human and veterinary uses.

The compositions containing the recombinant HMPV are administered to a host susceptible to or otherwise at risk for HMPV infection to enhance the host's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of recombinant HMPV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per host, more commonly from about 10^4 to 10^6 PFU virus per host. In any event, the formulations should provide a quantity of modified HMPV of the current disclosure sufficient to elicit a detectable immune response in the host patient against the subject pathogens.

The recombinant HMPV can be combined with viruses of other HMPV serotypes or strains to elicit a desired immune response against multiple HMPV serotypes or strains. Alternatively, an immune response against multiple HMPV serotypes or strains can be achieved by combining protective epitopes of multiple serotypes or strains engineered into one virus, as described herein. Typically when different viruses are administered they will be in admixture and administered simultaneously, but they can also be administered separately. Immunization with one strain may immunize against different strains of the same or different serotype.

In some instances it may be desirable to combine the recombinant HMPV immunogenic compositions with immunogenic compositions that induce immune responses to other agents, particularly other childhood viruses. In another aspect of the current disclosure the recombinant HMPV can be employed as a vector for protective antigens of other pathogens, such as RSV, by

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incorporating the sequences encoding those protective antigens into the recombinant HMPV genome or antigenome that is used to produce infectious virus, as described herein.

In all subjects, the precise amount of recombinant HMPV administered, and the timing and repetition of administration, will be determined using conventional methods based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^6 PFU virus per patient. In any event, the formulations should provide a quantity of attenuated recombinant HMPV sufficient to effectively stimulate or induce an anti- HMPV or other anti-pathogenic immune response, for example, as can be determined by hemagglutination inhibition, complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As with administration to chimpanzees, the attenuated virus of grows in the nasopharynx of recipients at levels approximately 10-fold or more lower than wild type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated virus.

In neonates and infants, multiple administrations can be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain an immune response against native (wild type) HMPV infection. Similarly, adults who are particularly susceptible to repeated or serious HMPV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, can require multiple immunizations to establish and/or maintain immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of immune response. Further, different candidate viruses may be indicated for administration to different recipient groups. For example, an engineered HMPV expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

HMPV-based immunogenic compositions can be combined with viruses expressing antigens of another subgroup or strain of HMPV to elicit an immune response against multiple HMPV subgroups or strains. Alternatively, the candidate virus can incorporate protective epitopes of multiple HMPV strains or subgroups engineered into one HMPV clone, as described herein.

The recombinant HMPV immunogenic compositions elicit production of an immune response that reduces or alleviates serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild type HMPV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination. Boosting of resistance

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by subsequent infection by wild type virus can occur. Following immunization, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild type virus *in vitro* and *in vivo*.

5 In one embodiment, recombinant HMPV candidates exhibit a very substantial diminution of virulence when compared to wild type virus that naturally infects humans. The virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus can still be capable of dissemination to nonimmunized individuals. However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the immunized host do not occur.

10 The level of attenuation of recombinant HMPV candidates can be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild type HMPV or other attenuated HMPV which have been evaluated as candidate strains. For example, the attenuated virus will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a
15 chimpanzee, compared to the levels of replication of wild type virus, for example, 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, a useful candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses must be sufficiently infectious and immunogenic in humans to elicit a desired immune response in
20 immunized individuals. Methods for determining levels of HMPV in the nasopharynx of an infected host are well known in the literature and facilitated by the methods and compositions disclosed herein.

Levels of induced immunity provided by the immunogenic compositions of the current disclosure can also be monitored by measuring amounts of neutralizing secretory and serum
25 antibodies. Based on these measurements, dosages can be adjusted or immunizations repeated as necessary to maintain desired levels of immune response. Further, different candidate viruses can be advantageous for different recipient groups. For example, an engineered recombinant HMPV strain expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

30 In another embodiment, the recombinant HMPV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the recombinant HMPV genome or antigenome incorporates a sequence that is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls HMPV expression. The infectious recombinant HMPV produced by coexpressing the recombinant
35 HMPV genome or antigenome with the N, P, L and other desired HMPV proteins, and containing a sequence encoding the gene product of interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being

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5 treated. Recombinant HMPV is administered in an amount sufficient to result in the expression of therapeutic or prophylactic levels of the desired gene product. Representative gene products that may be administered within this method are preferably suitable for transient expression, including, for example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor suppressor genes, antisense RNAs, and other candidate antigens.

10 The following examples are provided by way of illustration, not limitation. These examples describe the development of a novel reverse genetics system for the recovery of HMPV from cDNA, and the use of this system for construction of novel recombinant HMPV candidates. Thus, the subject matter of the current disclosure is illustrated by the following non-limiting Examples.

EXAMPLES

15 The mononegaviruses generally have a genome that comprises a single negative-sense strand of RNA tightly encapsidated in a nucleocapsid. There is a virally encoded polymerase that associates with the nucleocapsid and directs transcription of the genome by a process that initiates at the 3' end and copies the linear array of genes by a sequential stop-start mechanism that yields subgenomic mRNAs. Replication involves the synthesis of a complete positive sense copy called the antigenome, which in turn serves as the template for producing progeny genomes. Newly synthesized viral glycoproteins are transported to and accumulate at the plasma membrane. Progeny virus is formed when the nucleocapsid associates with these areas of modified plasma membrane and acquires a lipid envelope by budding.

25 RSV is the leading cause of hospitalization for viral lower respiratory tract disease in infants and young children, followed by HPIV3 (Collins *et al.*, 4th ed. In "Fields Virology," D. M. Knipe, P. M. Howley, Eds., Vol. 1, pp. 1443-1485, Lippincott-Williams and Wilkins Publishers, Philadelphia, 2001; Crowe *et al.*, Vaccine 13:415-421, 1995; Marx *et al.*, J. Infect. Dis. 176:1423-1427, 1997). HPIV1 and HPIV2 are the principal etiologic agents of laryngotracheobronchitis (croup), and can also cause pneumonia and bronchiolitis (Chanock *et al.*, 4th ed. In "Fields Virology," D. M. Knipe, P. M. Howley, Eds., Vol. 1, pp. 1341-1379, Lippincott-Williams and Wilkins Publishers, Philadelphia, 30 2001). RSV and the PIVs also are important causes of respiratory tract disease in adults. The available evidence indicates that HMPV is also a significant agent of human respiratory tract disease, particularly in young infants and children but occurring in all age groups (Boivin *et al.* J. Infect. Dis. 186:1330-6, 2002).

35 HMPV replicates slowly and inefficiently in cell culture, rendering it relatively challenging to maintain and study in laboratory. In addition, trypsin reportedly must be present in the medium in order to activate infectivity of the virus, which complicates the lengthy incubations that are necessary

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to successfully propagate the virus. Indeed, the difficulty in propagating HMPV provides an explanation for why it previously escaped detection as an important respiratory pathogen despite several decades of widespread cultivation of respiratory tract specimens in cell culture in many hospitals and research facilities across the world.

5 Methods for the production of RSV and PIV are known in the art, and compositions to induce an immune response against these viruses have been described (see Durbin *et al.*, Virology 235:323-332, 1997; Skiadopoulos *et al.*, J. Virol. 72:1762-1768, 1998; Skiadopoulos *et al.*, J. Virol. 73:1374-1381, 1999; Tao *et al.*, Vaccine 19:3620-3631, 2001; Durbin *et al.*, J. Virol. 74:6821-6831, 2000; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application
10 Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), U.S. Provisional Application No. 60/059,385, filed September 19, 1997; U.S. Provisional Application No. 60/170,195 filed December 10, 1999; and U.S. Patent Application Serial No. 09/733,692, filed December 8, 2000 (corresponding
15 to International Publication No. WO 01/42445A2), each incorporated herein by reference). However, HMPV provides a contrasting situation in which there are no available reference reagents such as monoclonal antibodies or available reference viral strains, and no established methods for the detection and analysis of HMPV. Knowledge concerning the molecular biology of HMPV has remained rudimentary and was previously based largely on extrapolation from other viruses.
20 Because HMPV has only recently been identified and its characterization had been very incomplete, there were many challenges to generating suitably attenuated, immunogenic and genetically stable for use in immunogenic and diagnostic compositions. To facilitate these goals, it is necessary to produce recombinant infectious HMPV from cDNA to serve as a starting point for manipulations that include the development and staged introduction of predetermined attenuating mutations; the deletion,
25 modification or rearrangement of existing genes, the introduction of foreign genes, the swapping of protective antigens between strains, and the swapping of attenuating or other desired mutations between HMPV strains and between HMPV and other related viruses, and to provide additional tools to generate immunogenic compositions, vectors and immunization methods. The present disclosure describes the production of recombinant infectious HMPV from cDNA, and attenuating mutations
30 with this cDNA, as well as the specific deletions and substitutions within existing genes, the introduction of heterologous nucleic acid sequences, and additional methods and compositions that are of use in methods designed to generate an immune response against HMPV.

35

Example 1

Determination of a Complete Consensus Sequence for the Genome of HMPV

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The present Example provides the complete genomic sequence of a HMPV . The subject viral strain for this analysis is strain CAN97-83 (Peret *et al.*, J. Infect. Dis. 185:1660-3, 2002), hereafter referred to as strain 83 or CAN83. The sequence was determined by direct analysis of uncloned RT-PCR products of viral RNA and thus represents a consensus or majority sequence of a viable virus. The virulence, and hence wild type status, of this virus was confirmed by the ability to induce respiratory tract disease signs in chimpanzees following intranasal inoculation, as described in a following Example. The determination of a complete authentic wild type HMPV sequence is a necessary step in developing a system to produce HMPV of defined sequence and characteristics for use in immunogenic compositions and methods.

Cell Lines and Viruses

HEp-2 (ATCC CCL 23), Vero (ATCC CCL-81) and LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM I (Invitrogen GIBCO) supplemented with 5% fetal bovine serum and, in some instances, gentamicin sulfate (50 µg/mL). BSR T7/5 cells are baby hamster kidney 21 (BHK-21) cells that have been transformed and constitutively express T7 RNA polymerase (Buchholz *et al.*, J. Virol. 73:251-9). They were maintained in Glasgow MEM supplemented with glutamine and amino acids (Invitrogen) and 5% fetal bovine serum. The BSR T7/5 cells were subjected to geneticine (1 mg/ml) selection every second passage. Biological and recombinant HMPV were propagated in Vero, LLC-MK2, or BSR T7/5 cells in the absence of serum and the presence of 5 µg/ml of trypsin.

Virion RNA Isolation

Confluent monolayers of LLC-MK2 cells were infected with HMPV and incubated at 32°C in the presence of 5 µg/ml trypsin. 10-14 days post-infection, clarified supernatants were harvested. Virion RNA (vRNA) was isolated directly from the clarified supernatants using the QIAamp viral RNA purification kit (Qiagen) according to the manufacturer's instructions. Alternatively, virus was purified by sucrose gradient centrifugation. Supernatant and infected cells were harvested by scraping with a rubber policeman, mixed with HEPES buffer and MgSO₄ at a final concentration of 50 mM and 0.1 M, respectively, and then submitted to three cycles of freezing and thawing. The mixture was clarified by low-speed centrifugation and the virus was pelleted by centrifugation at 7000 rpm overnight at 4°C. The virus pellet was resuspended in OptiMEM (Invitrogen) containing 50 mM of HEPES buffer and 0.1 M of MgSO₄. The viral suspension was layered on a 30 to 60% sucrose gradient and was centrifuged for 90 min at 26,000 rpm in an SW28 rotor. The upper virus-containing fraction was collected, diluted in TEN (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 1 mM EDTA) and virus particles were pelleted by centrifugation at 25,000 rpm in an SW28 rotor for 90 min. The virus pellet was resuspended in TEN and vRNA was isolated directly from an aliquot using the RNeasy kit (Qiagen) according to the manufacturer's instructions.

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Reverse Transcription, Polymerase Chain Reaction and Nucleotide Sequencing

vRNA was reverse transcribed using specific primers generated from the published partial sequence of HMPV strain 00-1. In most cases these failed to yield RT-PCR products, presumably reflecting nucleotide sequence differences between these two strains. Therefore additional primers were designed based on the regions between ORFs in the 00-1 sequence that, by analogy to other mononegaviruses, would likely contain cis-acting signals that would be more highly conserved between HMPV strains. This strategy provided RT-PCR products for strain 83 that, upon sequence analysis, provided information for designing strain 83-specific primers. Purified RNA was mixed with 50 pmol of different primers in a final volume of 14 µl in water and incubated for 5 min at 70°C and 25 min at 60°C for denaturing and annealing steps. Then, 5 µl of 5X first-strand buffer II, 1 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 2 µl of 0.1 M dithiothreitol, 1 µl of RNaseOUT (40 U/µl; Invitrogen) and 2 µl of Superscript II reverse transcriptase (Invitrogen) were added to the reaction. Synthesis of cDNA by RT was conducted at 44°C for 1 h and 30 min at 51°C to minimize the formation of RNA secondary structures. PCR was carried out on the reverse transcribed cDNA product using 50 pmol each of specific forward and reverse primers, 5 µl of 10X Pfx amplification buffer, 1 µl of dNTP mixture, 3 µl of MgSO₄ and 1 µl of Platinum Pfx DNA polymerase in a total volume of 50 µl. The PCRs were conducted on a Peltier Thermal Cycler (PTC-200; MJ Research) as follows: 3 min at 94°C and then 40 s at 94°C, 40 s at 55°C and 1 min/Kb at 68°C for 30 cycles and a final extension reaction for 7 min at 68°C. RT and PCR primers designed from the 00-1 strain sequence to prime within ORFs usually were not successful generating RT-PCR products from the 83 strain, presumably reflecting sequence differences relative to the 00-1 strain. Therefore, primers were designed from the semi-conserved sequence motifs flanking the viral ORFs (see Figure 8), which resulted in the successful generation of cDNA. The nucleotide sequences of cDNA products were determined by direct sequence analysis of the RT-PCR products using a ABI 3100 sequencer with the Big-Dye terminator ready reaction kit v1.1 (Applied Biosystems). Finally, the sequence was assembled from overlapping RT-PCR products.

To map and sequence the 3' end of the HMPV genome, vRNA was converted into cDNA and amplified using the 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Inc.) as specified by the manufacturer. Briefly, vRNA was polyadenylated at its 3'-end using poly A polymerase (Invitrogen, Inc.) followed by first-strand cDNA synthesis primed with oligo (dT) and PCR using an HMPV specific reverse primer and a forward UAP primer supplied with the kit. Then, RACE products were used as template for a nested-PCR using an internal HMPV specific reverse primer and the AUAP primer supplied with the kit to improve the specificity of the reaction. Finally, the amplified cDNA nested-PCR products were sequenced directly.

To map and sequence the 5' end of the HMPV genome, vRNA was processed by first-strand cDNA synthesis, terminal transferase tailing, and PCR amplification as specified by the 5' RACE System for Rapid Amplification of cDNA 5' end Version 2.0 (Invitrogen, Inc.) using the AAP primer

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supplied with the kit. Then, the amplified cDNA RACE products were used as template for a nested-PCR using an internal HMPV specific primer and the AUAP primer supplied with the kit to improve the specificity of the reaction. Finally, the amplified cDNA nested-PCR products were sequenced directly.

5 The 5' and 3' ends of the HMPV genome also were confirmed by genomic RNA ligation and RT-PCR followed by sequencing. Briefly, vRNA, isolated from sucrose gradient purified virus using the RNeasy kit (Qiagen), was ligated in presence of 10 U of T4 RNA ligase (Epicentre) for 3 h at 25°C and 3 h at 37°C. A cDNA corresponding to the junction of the ligated ends was amplified by
10 RT-PCR using HMPV specific primers. The RT-PCR products were used as template for a nested-PCR using HMPV internal specific primers to improve the specificity of the reaction. Finally, the nested-PCR products were cloned into a blunt vector (pSTBlue-1; Novagen) and 20 clones were sequenced, thereby confirming the length, content and sequence of the two previously-
uncharacterized ends of the HMPV genome.

 The complete sequence of HMPV strain 83 was determined to be 13335 nucleotides in
15 length, and is set forth in Figures 37A-37D. This sequence was determined directly from uncloned RT-PCR products and thus represents the authentic majority, consensus sequence of a virulent, wild type virus. This discovery provides a necessary step in the development of a reverse genetics system for recovering complete infectious virus and recombinantly engineered derivatives from cloned cDNAs. A partial sequence had been published representing a portion of the genome of HMPV strain
20 00-1 (van den Hoogen *et al.*, Virology 295:119-132, 2002).

 A genomic map for HMPV 83 is shown in FIG. 1. Compared to RSV, the prototype pneumovirus, HMPV lacks the NS1 and NS2 genes and contains a significant difference in gene order. Specifically, the SH-G gene pair precedes the F-M2 gene pair in RSV, whereas SH-G follows F-M2 in HMPV. Identification of HMPV genes was made based on the presence of ORFs whose
25 predicted protein products exhibit significant amino acid sequence relatedness with counterpart ORFs in RSV (N, P, M, F, M2-1 and L) or, in cases where sequence relatedness was unclear, gene identifications were made by genome position and by the characteristics of the predicted protein (M2-2, G, SH). In these analyses, RSV was chosen for comparison because all of its proteins have been directly identified and in most cases functions have been assigned. In a number of cases, the size of
30 the predicted HMPV protein was substantially different from that of its RSV counterpart. For example, the putative HMPV SH protein was 180% larger than that of RSV (179 amino acids compared to 64) and the HMPV G protein was 27% shorter (219 amino acids compared to 298) and lacked certain characteristic structural features such as the conserved cysteine noose (Johnson *et al.*, Proc. Natl Acad.Sci. USA 84:5625-9, 1987) and CX3C chemokine domain (Tripp *et al.*, Nat. Med.
35 2:732-8, 2001). Even the putative HMPV P and L proteins exhibited significant differences in length, being 22% larger and 7% shorter, respectively, than the proposed RSV counterparts.

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The ends of the HMPV genome were mapped and sequenced. The deduced sequences of the 3' leader and 5' trailer regions are shown in FIG. 2. This analysis ruled out the possibility that one or more additional genes might follow the L gene or precede the N gene, comparable to the NS1 and NS genes of RSV. Comparison of the leader and trailer regions of HMPV 83 with that of APV

(Randhawa *et al.*, J. Virol. 71:9849-9854, 1997) and RSV (Mink *et al.*, Virology 185:615-624, 1991) showed a high degree of sequence identity between the first 22-23 nucleotides at each end, consistent with the idea that these conserved sequences represent important functional domains. In the case of RSV, it was directly demonstrated that the first 11 nucleotides of the RSV genome represent the core promoter essential for both transcription and RNA replication (Fearn *et al.*, J. Virol. 76:1663-1672, 2002).

Thus, the published partial sequence for strain 00-1 appears to lack the core element of both the genomic and antigenomic promoters, and probably lacks any HMPV promoter whatsoever. It is noteworthy that, within the first 12 nucleotides at either end of the HMPV genome, there were at least one (3' leader) or two (5' trailer) sequence differences compared to the other pneumoviruses. Thus, the expedient of using the termini of APV or RSV to supplement the incomplete HMPV 00-1 sequence by constructing a chimeric molecule would not have yielded an authentic HMPV sequence at either end. While the functional significance of these particular differences have yet to be fully elucidated, studies with RSV showed that any point mutations within the genomic promoter affected transcription or RNA replication or both, and in most cases these effects were severe (Fearn *et al.*, J. Virol. 76:1663-1672, 2002). In any event, for the development of a genetic system to engineer recombinant HMPV for use in immunogenic compositions, it is critical to begin with a verified authentic wild type sequence, as provided herein. This sequence was used as a starting point; confirmation that the sequence indeed is authentic and encodes a functional virus is provided by the recovery of infectious, wild type-like recombinant virus. This demonstration is provided in examples below.

Another important feature of mononegavirus genomic RNA is that the 3' and 5' ends typically exhibit terminal complementarity, meaning that the two ends are complementary when aligned in an antiparallel fashion. This reflects the presence of a conserved sequence at each end that probably reflects a conserved promoter core. This terminal complementarity is shown for HMPV 83 and APV in FIG. 3. For typical mononegaviruses, the two ends exhibit a high degree of complementarity for at least the first 12 nucleotides and often more, and typically within this region the complementarity is exact or has at most one mismatch. As shown in FIG. 3, the APV genome indeed has an exact match for the first 13 nucleotides whereas, surprisingly, the HMPV 83 genome has two adjacent mismatches. This further illustrates that extrapolation from other pneumoviruses to develop a HMPV genetic system based on an incomplete sequence could not have yielded an authentic HMPV sequence.

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The sequence of HMPV 83 contained a number of differences compared to that published for strain 00-1. As examples, FIG. 4 shows that the SH ORF terminated four codons earlier in the 83 sequence than in that for strain 00-1, yielding a shorter SH protein. Also, the G ORF in the HMPV 83 sequence contained a deletion that shortened the G protein by 17 amino acids, and the intergenic
5 region between the F and M2 genes was 13 nucleotides for 83 instead of 41 nucleotides. The significance of differences such as shown in FIG. 4 can be directly evaluated with the reverse genetics system of this current disclosure.

The percent amino acid sequence identity between the predicted proteins of HMPV strain 83 and those of other pneumoviruses is shown in FIG. 5. It should be noted that a subsequent Example
10 provides a more extensive comparison between strain 83 and another HMPV strain, 75, that forms part of the current disclosure, demonstrating the extent of sequence diversity between and within the proposed HMPV subgroups. It is noteworthy that, among all of the pneumoviruses shown in FIG. 5, reverse genetics systems for the recovery of complete infectious virus have been reported only in the case of bovine RSV (BRSV) and human RSV subgroup A. This is despite that fact that PVM has
15 been known for almost 65 years and has been the subject of extensive molecular analysis beginning in the late 1980's, and despite the fact that APV has been known for almost 25 years and also is the subject of molecular analysis. The fact that reverse genetics systems are not yet available despite considerable interest in their development illustrates the difficulty of producing such systems. As has
20 already been noted, HRSV and BRSV differ from HMPV with regard to the lack of two genes (NS1 and NS2) and the rearrangement of other genes (SH and G). HRSV and BRSV also differ extensively from HMPV with regard to amino acid sequence identity of the predicted HMPV proteins. Three of the potential HMPV proteins (SH, G, and M2-2), share only 6-15% sequence identity with their putative HRSV counterparts, whereas low sequence identity values (for example, less than 25% identity) are generally considered at best to correspond to remote homologs or, alternatively, to
25 proteins that are unrelated. Conversely, the highest value for percent identity is only 46%, in the case of the L protein. Thus, HMPV is quite distinct from any virus for which a reverse genetics system for the recovery of virus has been developed. It should be further noted in this context that the function of a protein in HMPV cannot be reliably predicted by extrapolation from results obtained for a related protein of a related virus, not even for a closely related virus. Briefly, there is initial
30 uncertainty in deducing proteins from nucleotide sequence. For example, despite the reported existence of an M2-2 ORF in APV, the available evidence suggests that this ORF is not expressed (Ahmadian *et al.*, J. Gen. Virol. 80:2011-2016, 1999), whereas it clearly is expressed in RSV and has been shown to have a role in regulating RNA synthesis (Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Jin *et al.*, J. Virol. 74:74-82, 2000). Thus, the presence of an ORF
35 is not proof alone of significance. As another example, in the Filovirus family, both Ebola and Marburg viruses express a protein called VP30 that is highly related between the two viruses. However, these two proteins have different functions (Weik *et al.* J. Virol. 76:8532-9, 2002;

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Muhlberger *et al.*, J. Virol. 73:2333-42, 1999). These are but two examples of the uncertainty in predicting the existence and function of a protein based on nucleotide sequence alone. This highlights the importance of direct, relevant assays as provided hereon for the demonstration of protein function. These are presented in the current disclosure in two forms: (i) the development of a mini-replicon that is capable of transcription and replication when supplied with appropriate viral proteins, and importantly (ii) the development of a system for the recovery of complete infectious virus completely from cDNA, which provides the basis for analysis of the function of all elements of the genome and its encoded RNA and protein products and infectious virus.

The amino acid sequence of the predicted M protein of HMPV 83 was used to estimate the phylogenetic relationships between HMPV and other members of the Paramyxovirus family, as illustrated in FIG. 6. As shown in this figure, the Paramyxovirus family consists of two subfamilies Pneumovirinae and Paramyxovirinae, herein referred to as pneumoviruses and paramyxoviruses, respectively. These subfamilies are further divided into genera, the genus of HMPV being the Metapneumoviruses. A system for producing infectious recombinant virus did not exist previously for any member of this genus. This analysis provided further evidence of evolutionary distance and distinction from pneumoviruses such as RSV and, in particular, from paramyxovirus such as Sendai, measles, mumps, human parainfluenza, and Newcastle disease viruses that constitute the more well-known and more extensively-characterized members of the Paramyxovirus family.

Example 2

Assembly of a Full-Length HMPV Antigenomic cDNA Clone Containing a GFP Transcription Cassette and Recovery of Infectious Recombinant HMPV

The complete, authentic, consensus sequence (FIGS. 37A-37D) determined for HMPV strain 83 provided the basis for designing a system for producing infectious recombinant virus. FIG. 7 illustrates the construction of a cDNA clone encoding the complete antigenome of HMPV strain 83 designed from the complete consensus sequence. Three separate cloned subgenomic fragments were created: fragment 1 contains the putative N, P and M genes and is bordered on the upstream (left) side by an added promoter for bacteriophage T7 RNA polymerase (T7pr) and on the right hand side by an *NheI* site that was added to the putative N-F intergenic region as a marker. The T7 promoter was designed to add three nonviral G residues to the 5' end of the antigenome, a configuration chosen to improve the efficiency of the T7 promoter. Fragment 2 included the putative F, M2, SH and G genes and is bordered on the upstream side by the added *NheI* site and on the downstream side by a naturally-occurring *Acc65I* site. The *NheI* site serves as a marker to distinguish between cDNA-derived and biologically-derived HMPV. Fragment 3 consists of the putative L gene followed by half of the hepatitis delta virus ribozyme (HDVribo) bordered by an *RsrII* site that occurs naturally within the ribozyme. The sequence of each cloned cDNA fragment was confirmed. The vector for cloning and expressing the HMPV 83 antigenome cDNA is pBSKSII, which contains the hepatitis delta virus

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ribozyme followed by a terminator for T7 RNA polymerase (T7t) and was derived from vector p3/7 that was used previously for the recovery of recombinant HPIV1, HPIV2 and HPIV3 (Durbin *et al.*, Virology 235:323-332, 1997; Schmidt *et al.*, J. Virol. 74:8922-9, 2000; Skiadopoulos *et al.*, J. Virol. 77:270-9, 2003; Newman *et al.*, Virus Genes 24:77-92, 2002). This vector was modified by the
5 insertion of a polylinker containing *Ava*I, *Aat*II, *Nhe*I, and *Acc*65I sites, which in turn served to accept the fragment 1, 2 and 3 restriction sites. Specifically, the polylinker consists of the following sequence, *CCCGGGGACGTCCTAGCTAGCTAGGGTACCCCGCTCGAGCGGTCCG* (SEQ ID NO: 33; *Sma*I and *Rsr*II sites italicized), and was inserted between the *Sma*I and *Rsr*II sites of the vector p3/7 of Durbin *et al.* (Durbin *et al.*, Virology 235:323-332, 1997). The final cDNA encodes
10 the complete 13335-nucleotide HMPV 83 antigenome containing three added nonviral G residues at the 5' end. The complete pHMPV-83 recombinant plasmid ("antigenome plasmid") contains 16333 bp.

It was anticipated that the poor growth and lack of established reference reagents and assays for HMPV could complicate the recovery and identification of recombinant HMPV recovered from
15 cDNA. Therefore, the strategy was adopted to modify the antigenomic cDNA to insert a foreign marker gene encoding a detectable label, whereby expression of the marker gene could be readily monitored. The gene that was chosen encodes the well-known marker or label GFP, a cnidarian protein whose presence can be visualized by green fluorescent emission when observed under a fluorescent microscope. The GFP cDNA was the Enhanced GFP cDNA of Clontech, Inc.
20 Expression of a detectable marker in this manner, directly linked with HMPV production, makes it possible to monitor expression of GFP by recombinant HMPV in living cells without loss of viability or sterility, and to thereby detect and quantify the recovery of rHMPV at all stages of transfection and passage.

Among the obstacles to this strategy were the lack of direct information on HMPV
25 transcription and the lack of direct mapping of gene boundaries. It therefore was necessary to identify sequence that, when attached to the foreign ORF, would render it capable of being expressed during the HMPV transcriptional program. It also was necessary to identify a site within the HMPV genome that could accommodate the insertion of a foreign gene without disrupting HMPV growth. The transcription of a typical mononegavirus genome initiates at or near the 3' end and proceeds by a
30 sequential start-stop mechanism by which the individual genes are transcribed into individual, separate mRNAs. Typically, mononegavirus transcription is guided by short sequence motifs that flank the viral genes. For example, the genes of RSV, which is the most thoroughly characterized pneumovirus, contain a highly-conserved 10-nucleotide GS signal on the upstream end of each gene and a semi-conserved 12- to 13-nucleotide GE signal at the downstream end of each gene. These
35 motifs were first noticed when RSV mRNAs were isolated from infected cells and their termini were directly mapped and sequenced. It is thought that the GS signal directs initiation of transcription of the individual gene, and the GE signal directs polyadenylation and termination. The polymerase

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remains bound to the template and crosses the intergenic region to resume transcription at the next gene. In the case of RSV, the analysis of the mRNA ends showed that the arrangement of genes was unexpectedly complex. Specifically, the GS signal of the L gene was found to differ from that of the others, and furthermore was found to be located within its upstream neighbor rather than downstream of it. As another example, the M2-1 and M2-2 ORFs were found to be contained in a single mRNA rather than two. Also, there was no consistency of spacing of the RSV GS and GE signals with respect to the ORFs, such that there were instances where the GS signals overlapped the ORFs or, alternatively, were separated from the ORFs by as many as 85 nucleotides. Similarly, certain RSV GE signals overlapped the respective ORF or were separated by as many as 173 nucleotides. Furthermore, the spacing between the RSV genes was irregular, ranging from the overlapped genes mentioned above to ones separated by as many as 56 nucleotides. This indicates the uncertainty of predicting gene boundaries based on the locations of putative ORFs and potential sequence motifs. However, the methods of this current disclosure provide for the direct identification of appropriate transcription signals and insertion sites.

The complete sequence of HMPV strain 83 was examined for potential GS and GE motifs, as shown in FIG. 8. These motifs were identified on the basis of being located between ORFs and by exhibiting partial sequence conservation among themselves. For example, the putative HMPV GS motif has the sequence GgGAcAAgTgaaaATG, where the nucleotide assignments in upper case are conserved in each of the putative genes. In sharp contrast to RSV, the putative GS signals were found in close proximity to the start of their respective putative ORFs, with the putative initiation ATG codon located at positions 14-16 relative to each putative GS signal. It should be noted that the L ORF was preceded by two potential GS sequences, either of which was a reasonable fit for the proposed consensus sequence. One of these is shown in FIG. 8, while the second is located 18 nt upstream (7102-GGGCAAAACAGCATCC). Typically mononegavirus genes each have a single GS site, and it is not known which one of these is functional for the HMPV L gene. However, this can now be readily determined by the methods of this current disclosure, by mapping the 5' end of the putative L mRNA by RT-PCR, or by testing each potential GS signal for functionality in a mini-replicon system, or by destroying each potential signal in the complete antigenome and evaluating each mutant for virus recovery and efficiency of growth.

The putative GE motif has the sequence AGTtaattaAAAA, where the upper case assignments are conserved in each of the putative genes. The limited number of highly-conserved nucleotide assignments in these motifs made their identification speculative, particularly since not a single HMPV signal had been directly identified to use as a benchmark. While these conserved motifs appear to contain the core of the GS and GE signals, it would not be surprising to find that either sequence as described here might be shortened at one or both ends by one or several nucleotides without complete loss of activity, or that one or more additional nucleotides added to either side might affect the activity of either signal. As an indication of the uncertainty of deducing transcription

signals from sequence data alone, van den Hoogen *et al.* noted two different types of consensus sequences between genes, one of which corresponds to that noted above (van den Hoogen *et al.*, Virology 295:119-132, 2002). In this regard, these authors concluded that they could clearly identify this particular consensus sequence for two genes, F and L (although, as noted above, there would appear to be two candidates to be the GS signal for the L gene), found “variants” in several other genes, failed to detect a counterpart for the G gene, and noted that “another [different] repeated sequence...was found downstream of each of the hMPV ORFs except G”, raising further uncertainty as to which, if any, of these sequence motifs might function as a transcription signal. Thus, it was important to directly identify transcription signals that could render a foreign sequence competent for expression by HMPV.

In order to directly identify sequence signals that can confer gene expression, the 16-nucleotide putative GS sequence and 13-nucleotide putative GE sequence of the N gene were attached to a cDNA containing the GFP ORF. In this configuration, the ATG present in the GS signal replaced that of the GFP ORF. As shown in FIG. 9, this transcription cassette was inserted into the HMPV antigenomic cDNA following nucleotide 41, resulting in an antigenome in which the GFP gene was placed first in the 5' to 3' order. In the corresponding HMPV-GFP genome, the GFP gene would be the most promoter-proximal of the genes. The complete sequence of the antigenomic rHMPV-GFP cDNA (FIGS. 38A-38D) and flanking plasmid regions was confirmed by sequence analysis.

Initially four of the ORFs of HMPV, namely the putative N, P, L and M2-1 ORFs, were selected to be expressed in separate plasmids to complement recovery. This initial recovery protocol focused on proteins predicted to be “internal” proteins (as opposed to components of the envelope). Examination of the HMPV genome identified ORFs whose potential products share 41%, 31%, 36%, and 46% identity with the RSV N, P, M2-1 and L proteins, respectively (FIG. 5), and these ORFs were selected as candidates for inclusion. The putative M2-1 HMPV ORF also was included in the recovery because it is a possible internal protein, and studies with RSV, Ebola virus and Marburg virus indicate that possible M2-1 counterparts can be involved in RNA synthesis. In this regard it is noted that the presumed M2-1 counterparts appear to play different roles in Ebola and Marburg viruses despite their high degree of relatedness (Weik *et al.* J. Virol. 76:8532-9, 2002; Muhlberger *et al.*, J. Virol. 73:2333-42, 1999). Also, the putative HMPV M2-1 protein contains a Cys-7/Cys-15/Cys-21/His-25 motif (numbered according to the complete deduced HMPV M2-1 amino acid sequence, designated herein the Cys3-His1 motif) that is a potential zinc binding domain that might indicate a role in interacting with nucleotides or nucleic acid. On the other hand, the putative M2-2 ORF was not included in the initial recovery system, and there is evidence from APV that a seemingly-corresponding M2-2 ORF is not expressed (Ahmadian *et al.*, J. Gen. Virol. 80:2011-2016, 1999).

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The ORFs for the G, F and SH proteins of HMPV were not included in the initial recovery system, because these ORFs were predicted to encode transmembrane surface proteins--based on the presence of putative hydrophobic signals and anchors in the deduced amino acid sequences. Notably, the predicted HMPV F protein shares 36% amino acid sequence relatedness to that of RSV. The percent amino acid identity for the putative G and SH proteins between HMPV and RSV are 15% and 6%, respectively. Despite this substantial departure in primary structure, other features of the amino acid sequences, such as the presence of potential glycosylation sites support the identification of these proteins herein. It is generally acknowledged in the field that the envelope proteins do not have a known role in genome transcription and RNA replication. This does not preclude the possibility that the expression of these additional ORFs might facilitate recovery, perhaps by increasing the efficiency of virion assembly. However, an important aspect of the current disclosure involves identification of a minimal complement of support plasmids necessary for HMPV recovery. Once minimal recovery elements were shown to be successful, additional embodiments can now be constructed and evaluated to optimize the efficiency of recovery. For example, although the expression of the M2-1 ORF from a separate plasmid is shown herein to be non-essential for recovery, it may nonetheless be a desired complement that could function to influence transcription or RNA replication or some other aspect of HMPV replication. Accordingly, expression plasmids were constructed in which the N, P, L and M2-1 ORFs were placed under the control of a T7 transcription promoter and terminator.

Support plasmids encoding putative HMPV nucleocapsid and polymerase proteins were prepared as follows (FIG. 10): to prepare a support plasmid expressing the putative N protein, vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative N ORF (nucleotides 55 to 76 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative N ORF (nucleotides 1239 to 1220 in the HMPV genome). The PCR product was digested with *XhoI* and subjected to a partial digestion with *AflIII*, which was done because the putative N ORF contains a naturally-occurring *AflIII* and a partial digest would yield some product that had been cleaved at the terminal site and not at the internal site. The products were into pTM1 (Durbin *et al.*, Virology 235:323-332, 1997; Durbin *et al.*, Virology 234:74-83, 1997; Elroy-Stein *et al.*, Proc. Natl. Acad. Sci. USA. 86:6126-30, 1989) that was digested with *NcoI* (which leaves overhangs compatible with *AflIII*) and *XhoI*, and clones containing the complete putative N ORF were selected and the sequence confirmed.

To construct a support plasmid containing the putative P ORF of HMPV (pT7-P) (FIG. 10), vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative P ORF (nucleotides 1263 to 1279 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative P ORF (nucleotides 2147 to 2132 in the HMPV genome). The PCR product was digested with *AflIII* and *XhoI* and cloned into pTM1 that was digested with *NcoI*

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and *Xho*I. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis..

A comparable putative M2-1 support plasmid for HMPV (pT7-M2-1) was generated (FIG. 10). vRNA was subjected to RT-PCR using a positive sense primer designed to hybridize at beginning of the putative M2-1 ORF (nucleotides 4724 to 4740 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative M2-1 ORF (nucleotides 5287 to 5272 in the HMPV genome). The PCR product was digested with *Afl*III and *Bam*HI and cloned into pTM1 that was digested with *Nco*I and *Bam*HI. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis.

A support plasmid containing the putative L ORF of HMPV (pT7-L; FIG. 10) was made by RT-PCR amplification of two overlapping fragments and cloned separately into pTM1 vector. The first fragment was amplified by RT-PCR using a positive sense primer designed to hybridize at the beginning of the putative L ORF (nucleotides 7133 to 7157 in the HMPV genome) and a reverse primer designed to hybridize downstream to a unique *Avr*II (nucleotides 10892 to 10897 in the HMPV genome) restriction site (nucleotides 11037 to 11018 in the HMPV genome), and the second using a positive sense primer designed to hybridize upstream to the unique *Avr*II site (nucleotides 10845 to 10866 in the HMPV genome) and a reverse primer designed to hybridize at the end of the putative L ORF (nucleotides 13150 to 13124 in the HMPV genome). Then, the first fragment was treated by *Bam*HI and the Klenow fragment of the *E. coli* polymerase I to generate a blunt end, digested by *Avr*II and cloned into pTM1 that was treated by *Nco*I and Klenow enzyme and digested by *Avr*II. Finally, the second fragment of the putative L ORF was digested by *Avr*II and *Xho*I and cloned in the intermediate construct digested by the same enzymes, leading to the final construct pT7-L. In the final cloned recombinant plasmid, the sequence of the HMPV insert and flanking plasmid regions was confirmed by sequence analysis.

To provide an initial functional evaluation of these support plasmids, a mini-replicon system was developed. A cloned cDNA was constructed so that its expression from a T7 promoter yielded a negative-sense mini-replicon RNA, or minigenome, that contained, in 3'-to 5' order: the leader end of the HMPV genome and adjoining putative N GS signal followed by a negative sense copy of the ORF encoding bacterial chloramphenicol acetyl transferase (CAT), followed by the putative L GE signal and the trailer end of the HMPV genome. The correct 3' end was generated by a HDV ribozyme as described above. In this minigenome, the CAT gene would be under the control of putative HMPV GS and GE signals, and would be expressed as a subgenomic mRNA. The minigenome plasmid was transfected into HEp-2 cells together with the N, P, M2-1 and L support plasmids with simultaneous coinfection by vTF-7, a vaccinia virus recombinant that expresses T7 RNA polymerase. 48 h post transfection, intracellular RNA was isolated and analyzed by Northern blot hybridization using a double stranded probe specific to the CAT cDNA. This analysis demonstrated that coexpression of the N, P and L genes with the minigenome plasmid yielded RNA

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species of the appropriate size to represent progeny minigenomes, antigenomes, and subgenomic CAT mRNA. In this assay, the further addition of the M2-1 gene did not alter the profile of RNA products. This indicated that in this assay N, P and L coexpression was sufficient to reconstitute RNA replication and transcription. These findings are in sharp contrast to RSV, where the expression of M2-1 is necessary to achieve efficient synthesis of mRNA (Collins *et al.*, Proc. Natl. Acad. Sci. USA 93:81-85, 1996; Fearn and Collins, J. Virol. 73:5852-64, 1999). These preliminary results do not rule out any role for M2-1 in HMPV RNA synthesis, since it is possible that M2-1 might have one or more functions that are not required for minigenome replication and transcription but might be essential to launch infection by complete recombinant virus. The current disclosure provides the methods to expeditiously evaluate the role of M2-1 (and M2-2) in viral recovery, gene expression, and replication.

To test the ability to generate infectious recombinant HMPV from cDNA, the full-length HMPV-GFP antigenome plasmid was cotransfected with the N, P, M2-1 and L plasmids into BSR T7/5 cells, which are baby hamster kidney-21 (BHK-21) cells that constitutively express T7 RNA polymerase (Buchholz *et al.* J. Virol. 73:251-59, 1999). Transfections were done in 6-well dishes with 5 µg of antigenomic plasmid, 2 µg each of N and P support plasmid, and 1 µg each of M2-1 and L support plasmid per well. Transfections were done with SuperFect (Qiagen) or Lipofectamine 2000 (Invitrogen) in medium without trypsin or serum. One day post transfection trypsin was added to 5 µg/ml. Also, to maintain the cells in an active state, they typically were split and reseeded at a 1:3 ratio one or two days post transfection. In another, alternative permutation, fresh trypsin was added one day post transfection, the cells were incubated for one or more hours, the cells were scraped into the medium, and the total suspension was passaged to fresh LLC-MK2 or Vero cells. Approximately 12-24 h later the medium was replaced with fresh medium containing 5 µg/ml trypsin (unless otherwise noted, 5 µg/ml trypsin was the concentration that was routinely used).

When examined by fluorescent microscopy on successive days post-transfection, green cells were visualized that initially consisted of scattered isolated cells and subsequently formed small foci that consisted of two or more cells and exhibited cytopathic effect consistent with HMPV. When the transfection monolayer was split into new cultures, single green cells were visualized within one day and developed over successive days into multicellular syncytia. The expression of GFP was monitored by fluorescent microscopy and photographed 8 days post-transfection. The nature of the cytopathology was indistinguishable from that produced by biologically-derived HMPV. The recovered virus replicated in LLC-MK2 cells with kinetics and cytopathogenicity consistent with HMPV.

The ability to monitor the infection in live cells without compromising sterility can be used to expeditiously examine conditions to improve growth. For example, it was found that the replenishment of trypsin by the addition of further amounts during incubation resulted in a greater number of infected cells. In a preliminary experiment, the addition of fresh trypsin to a concentration

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of 2.5 or 5 µg/ml at two-day intervals resulted in the highest level of GFP expression and virus spread. However, the higher level also was associated with increased syncytium formation, which has the potential to cause more rapid cell death and decreased yield. Further experimentation, combined with assay of released infectious virus (an assay that also is facilitated by the expression of the GFP marker), will serve to identify the condition of incubation and level of trypsin that is optimal for the production of infectious virus. The most optimal schedule found to date has been to add 5 µg/ml fresh trypsin at intervals of 2 or 3 days.

The expression of GFP by cells infected with the recovered virus provided evidence that the virus was cDNA-derived and was not contaminating biologically-derived HMPV. Further identification of the virus as recombinant HMPV was made in two ways, by immunofluorescence and by detection of the *NheI* restriction site marker. In the immunofluorescence assay, antibodies from hamsters that had been infected with biologically-derived HMPV reacted specifically with cells infected with the putative recovered rHMPV-GFP. For this assay, BSR T7/5 cells were transfected for the recovery of recombinant HMPV-GFP as described above. Four days later the cells were split again at a ratio of 1:3 and incubated on a coverslip for nine more days. Incubations were in the presence of 5 µg/ml trypsin. The cells were fixed with 80% acetone for 15 min at 4°C, followed by incubation with serum from hamsters that had been infected with HMPV, followed by incubation with goat antibodies that were specific to hamster IgG and had been labeled with the fluorescent tag Alexa-488. The same field of cells was visualized by confocal microscopy with visible light and under conditions for fluorescence. This demonstrated strong fluorescence that was specific to foci in HMPV-infected cultures.

In a related assay series, RT-PCR was performed to amplify nucleotides 2719 to 3894 (numbered according to their position in the wild type HMPV sequence exclusive of GFP) in the rHMPV-GFP genomic RNA (FIG. 12A). This RT-PCR procedure yielded a product of the expected size when the template was RNA from cells infected with biologically-derived HMPV or recombinant HMPV-GFP, but only in the latter case was the cDNA cleaved by *NheI*, confirming the presence of the added *NheI* site specific to the recombinant antigenomic cDNA from which the recombinant virus was derived. Amplification of RT-PCR products was dependent on the addition of RT, indicating that the template was indeed viral RNA and not contaminating DNA. Infectious recombinant HMPV-GFP also was recovered when the M2-1 support plasmid was omitted from the panel of support plasmids. Although these results suggest that M2-1 is not required for recovery, it is possible that M2-1 is expressed from the antigenome plasmid, as was found in the case of RSV (Collins *et al.* Virology 295: 251-255, 1999). Also, it may be that co-expression of M2-1 will have some qualitative or quantitative effect on recovery. This is suggested by the observation that, in a typical experiment, three times more infectious virus was recovered in the presence of M2-1. It is believed that the minimal complement of proteins required for recovery of HMPV is N, P and L.

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The expression of the inserted GFP coding sequence also was characterized by Northern blot hybridization. Cells were infected with biologically-derived HMPV83, rHMPV, rHMPV-GFP, or were mock infected, and total intracellular RNA was isolated three days later and analyzed by Northern blot hybridization with double-stranded DNA probes specific to the GFP or M genes. As shown in FIG. 12B, the GFP-specific probe hybridized only with RNA from rHMPV-GFP-infected cells (lane 4), and detected an abundant RNA band of the appropriate size to be the predicted 746-nt (exclusive of polyA) GFP mRNA transcribed from the inserted transcription cassette. This showed that the putative HMPV GS and GE transcription signals indeed functioned in the context of the foreign ORF to direct the efficient synthesis of a monocistronic GFP RNA. The GFP probe also hybridized to several larger RNAs that were of low abundance and appeared to represent GFP-N, GFP-N-P and GFP-N-P-M read-through mRNAs, as well as to a large, faint band that was of the appropriate size to contain rHMPV-GFP genome and antigenome RNA. The very low levels of read-through mRNAs indicated that termination at the end of the GFP transcription cassette was very efficient, and hence an HMPV GE signal indeed had been correctly identified. Analysis with the M-specific probe identified in each of the viruses bands of the appropriate sizes to be the predicted 853-nt monocistronic M mRNA as well as bands representing the P-M and N-P-M read-through mRNAs. The M probe also detected the genome and antigenome RNA band for each virus, which in the case of rHMPV-GFP would be 748 nt larger than HMPV83 or rHMPV due to the presence of the GFP transcription cassette (FIG. 12B, lanes 6, 7 and 8).

To test the ability to recover recombinant HMPV that lacked the GFP gene, plasmid expressing the HMPV antigenome depicted in FIG. 11 but lacking the GFP insert was transfected into BRS T7/5 cells together with N, P, M2-1 and L support plasmids. The recovery of recombinant HMPV was confirmed by the detection of viral antigen in single cells and in foci by indirect immunofluorescence assay as described above.

The recovered recombinant HMPV strain 83 was compared to the biologically-derived strain 83 isolate with regard to the efficiency of multi-cycle replication *in vitro*. LLC-MK2 cells were inoculated with 0.01 plaque forming units (PFU) per cell and incubated at 32°C in the presence of 5 µg/ml trypsin. Aliquots of the medium were taken at 24 h intervals and flash frozen. The aliquots were subsequently diluted in medium containing fresh trypsin and analyzed in parallel by plaque assay to determine the infectivity titer. As shown in FIG. 13, the recombinant HMPV replicated efficiently over the 10 day period tested. The efficiency of replication of rHMPV was essentially indistinguishable from that of its biologically-derived counterpart. The ability to recover a wild type-like virus entirely from cloned cDNA showed that the sequences used to construct these cDNAs are fully functional, and thus define an authentic, functional, prototypic wild type HMPV and its encoded proteins. This is noteworthy, since these viruses exhibit a high frequency of mutation (and thus a sequence cannot be assumed to be functional). The rHMPV antigenomic cDNA had been sequenced in its entirety following construction, as noted above, with the result that its sequence was confirmed

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to be exactly as designed. Thus, this experiment established (i) the exact genomic sequence of a prototypic HMPV, and (ii) conditions for recovering cloned cDNAs representing this sequence. This verified prototypic system provides the basis for the expedited recovery of other strains of HMPV, as well as for the systematic modification of HMPV via changes introduced into the cloned cDNA intermediate. Finally, the ability to precisely reconstruct a wild type recombinant HMPV indicates the robustness and precision of the methods disclosed herein. This is further demonstrated by the ability to recovery numerous engineered derivatives described in subsequent examples.

Next, recombinant HMPV-GFP was compared to rHMPV with regard to the efficiency of multi-cycle growth *in vitro* to evaluate the effect of the addition of a transcription cassette. As shown in FIG. 14, rHMPV-GFP replicated over the tested 10-day period with an efficiency that, at times, was reduced more than 10-fold compared to rHMPV. However, at the end of the 10 day period the final titers were very similar. Thus, a foreign insert can be accommodated by HMPV without a drastic effect on *in vitro* replication, such that a recombinant virus containing such an insert can be feasibly manufactured. It also is possible that such an insert will prove to be attenuated *in vivo*, as can now be expeditiously determined in experiments employing experimental animals that were identified as supporting HMPV replication in experiments described in a subsequent Example.

Example 3

The Development of Recombinant HMPV Derivatives Containing Pre-determined Mutations Specifying Desired Phenotypic Changes

The ability to produce infectious rHMPV from cDNA provides the basis for the planned introduction of mutations into infectious HMPV to develop recombinant viral candidates possessing desirable characteristics for use in immunogenic compositions, such as temperature-sensitivity, cold adaptation, host range restriction, improved replication *in vitro*, reduced reactogenicity, increased safety, increased antigen expression, increased genetic or phenotypic stability, broader immunogenic coverage, increased immunogenicity, and attenuation. Mutations might be devised that have advantages in other applications as well, such as to yield increased antigen expression and/or to facilitate preparation of purified viral protein. Each antigenome can be assayed for the ability to direct the recovery of infectious virus, and recovered mutant viruses can be evaluated for *in vitro* effects on growth and cytopathogenicity, temperature sensitivity, plaque morphology, and other characteristics. Appropriate mutant viruses can then be evaluated for growth efficiency, antigen expression, temperature sensitivity, plaque morphology, cytopathogenesis, attenuation, pathogenicity, tropism, genetic and phenotypic stability, immunogenicity, safety, and protective efficacy in cell culture and predictive animal models for HMPV activity in human subjects, including mice, hamsters, cotton rats, and non-human primates, as well as in clinical studies.

One type of desirable mutation involves the deletion of sequence from the HMPV genome. For example, each of the HMPV genes or ORFs or parts thereof can be systematically deleted either

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in its entirety or in part, either alone or in combination with other genes or ORFs, to obtain virus with improved properties, particularly for use within immunogenic compositions. Exemplary genes or ORFs include the SH, G, M2-2 and M2-1 ORFs, but can involve any gene or ORF or genome region or part thereof, including non-ORF sequences such as noncoding genes or extragenic regions or cis-acting signals in whole or in part. As an example of the strategy of deleting entire genes, antigenomic cDNAs were designed in which the putative SH and G ORFs, each with their surrounding set of putative GS and GE signals, were deleted singly or together (FIG. 15). To make these constructs, the wild type GFP was modified to insert *Bsi*WI and *Bsr*GI sites at positions 5459 and 6099 (numbered relative to the complete antigenome exclusive of GFP, a convention that will be followed throughout this document). These positions are within the M2-SH and SH-G intergenic regions, respectively. Cleavage at either of these two sites creates the overhang GTAC, which also is the case for the naturally-occurring *Acc*65I site located at position 6959, within the G-L intergenic region (FIG. 15). Thus, the presence of these three compatible restriction sites flanking the SH and G genes facilitate deletion of either or both genes by cleavage with the appropriate pairs of enzymes followed by religation. This resulted in cDNAs encoding rHMPV-GFP antigenomes that lacked the SH (Δ SH) or G (Δ G) genes or both (Δ SH/G). Each antigenomic cDNA was evaluated for the ability to direct the recovery of infectious virus in BSR-T7 cells in the presence of the N, P, L and M2-1 support plasmids. In each case, infectious virus was readily and successfully recovered. These could then be evaluated with regard to kinetics and efficiency of growth *in vitro* and in experimental animals to define advantageous properties including attenuation. The presence of the expected deletion in each virus was confirmed by RT-PCR analysis of the SH-G region of each recovered genome, as shown in FIG. 16. Also, limited nucleotide sequencing of the RT-PCR products confirmed the sequences at the junctions of the deletions.

The rHMPV-GFP Δ SH, Δ G and Δ SH/G mutants were compared with rHMPV-GFP and rHMPV with regard to the efficiency of multi-cycle replication LLC-MK2 cells following infection with an input of 0.01 PFU per cell (FIG. 17A). In this experiment, the gene-deletion mutants contained the GFP marker gene and thus should be compared to rHMPV-GFP. Remarkably, rHMPV-GFP containing the deletion of a single gene, either SH or G, replicated 3- to 10-fold more efficiently than rHMPV-GFP alone. Thus, these genes are not essential for replication *in vitro* and, indeed, their loss improved replicative fitness *in vitro*. This may reflect a growth advantage due to the shorter length of the genome and loss of a transcriptional unit, although it also is possible that the absence of the SH or G protein might somehow improve growth. This can readily be investigated by the methods disclosed herein by ablating translation of SH and/or G by making nucleotide substitutions that remove translational start codons and introduce termination codons, thereby ablating expression of the protein while maintaining the gene number, the genome length, and the number of transcribed mRNAs. rHMPV-GFP lacking both SH and G replicated with an efficiency similar to that of complete rHMPV-GFP, suggesting as one interpretation that whatever gain might be made from

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deleting genes and shortening the genome was counteracted by a slight loss of replicative fitness due to the absence of both SH and G. Thus, production of an HMPV immunogenic composition might be improved by deletion of SH or G, but deletion of both might not be optimal. Also, deletion of either gene might increase the ability of rHMPV to accommodate one or more added foreign genes. Given
5 the efficient replication of these viruses *in vitro*, it will now be possible to evaluate the effects of these gene deletions on HMPV replication, immunogenicity, and pathogenesis in experimental animals and human volunteers.

A second, parallel set of Δ SH, Δ G, and Δ SH/G mutants was made in an rHMPV strain 83 backbone lacking the GFP marker gene. Each of the viruses from this second set of mutants was
10 readily recovered and propagated *in vitro*. These could be evaluated with regard to kinetics and efficiency of growth *in vitro* and in experimental animals to define advantageous properties including immunogenicity and attenuation. While the presence of the GFP gene can facilitate characterization of mutant viruses *in vitro* and *in vivo*, the GFP gene preferably would not be included in candidates for developing immunogenic compositions. Hence, it is advantageous to be able to expeditiously
15 produce mutants with or without GFP, or both, as appropriate.

The set of rHMPV Δ SH and Δ G viruses lacking the GFP marker gene were evaluated for replication *in vivo* (FIG. 17B). Golden Syrian hamsters were infected intranasally with biologically-derived HMPV83, rHMPV, rHMPV Δ SH, rHMPV Δ G, or rHMPV Δ SH/G. Six animals from each group were sacrificed three or five days later and the nasal turbinates and lungs were harvested,
20 homogenized, and analyzed by limiting dilution to determine the viral titers (FIG. 17B). There were several instances where differences in titer were observed between days 3 and 5, the most notable being the higher titers of HMPV 83, rHMPV and rHMPV Δ SH in the lungs on day 3 versus day 5. However, in general the titers for the two days followed a consistent pattern. Recombinantly-derived HMPV replicated *in vivo* with an efficiency similar to that of its biologically-derived parent
25 HMPV83. Thus, rHMPV appeared to have wild type-like growth properties *in vivo* as well as *in vitro* and represents a suitable starting point for developing attenuated derivatives as candidate vaccines.

Interestingly, the replication of HMPV was not significantly reduced by deletion of the SH gene (FIG. 17B). Indeed, the Δ SH virus replicated marginally better than its wild type counterpart, perhaps due to the shorter genome length and reduced gene number. In contrast, deletion of the G
30 gene resulted in a 2.9 (day 5) to 3.2 (day 3) \log_{10} decrease in replication in the nasal turbinates, and a 0.3 (day 5) to 2.3 (day 3) \log_{10} decrease in the lungs. The rHMPV Δ SH/G double-deletion virus exhibited a similar marked reduction in virus titer. Thus, these mutant viruses are highly attenuated *in vivo*.

In order to evaluate possible immunogenicity and protective efficacy, the rHMPV,
35 rHMPV Δ SH, rHMPV Δ G and rHMPV Δ SH/G viruses were administered intranasally to additional hamsters (FIG. 17C). Serum samples were taken 27 days later and analyzed for the ability to neutralize HMPV infectivity *in vitro* (FIG. 17C). This showed that the highly attenuated Δ G and

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ASH/G viruses induced high titers of HMPV-neutralizing serum antibodies even though they were highly attenuated. On day 28 post immunization, the animals were challenged intranasally with wild type HMPV, and the animals were sacrificed three days later and nasal turbinates and lungs were harvested and virus titers were determined by limiting dilution (FIG. 17C). This showed that no challenge virus replication could be detected in animals that had been infected with rHMPV or rHMPV Δ SH. Interestingly, animals that had been infected with rHMPV Δ G or rHMPV Δ SH/G also did not have detectable challenge virus replication in the lungs, and only a low level of challenge virus replication was detected in the nasal turbinates. Thus, despite their strongly attenuated nature, the rHMPV Δ G and rHMPV Δ SH/G viruses were immunogenic and highly protective against HMPV challenge. These viruses represent promising vaccine candidates. In particular, it was remarkable that deletion of the G gene would yield a promising vaccine candidate, since the G protein is presumed to be an attachment protein important in initiating infection, and the corresponding deletion in HRSV yielded a virus that did not replicate in mice (Teng et al, Virology 289, 283-296, 2001) and was over attenuated in humans (Karron *et al.*, Proc. Natl. Acad. Sci. USA 94: 13961-13966).

The deletion or otherwise modification of intergenic regions of HMPV offers an important strategy for achieving improved phenotypic properties. For example, the intergenic regions of HMPV strain 83 range in length from 2 nt (N/P) to 190 nt (G/L). Previous work with RSV indicated that plaque size was decreased if a single intergenic region was longer than 100 nt in length (Bukreyev *et al.*, J. Virol. 74:11017-26, 2000). Two of the naturally-occurring intergenic regions of HMPV strain 83 are greater than 100 nt in length, namely SH/G (124 nt) and G/L (190 nt). Thus, reducing the length of these or other intergenic regions offers a strategy to improve replication *in vitro*, while increasing intergenic length offers a strategy for attenuating the virus, as desired.

As another example, recombinant HMPV was recovered in which the M2-2 ORF was silenced (FIG. 18A). The proposed M2-1 ORF initiates at nucleotide position 4724 and terminates at position 5287. The proposed M2-2 ORF potentially initiates at positions 5235 or 5247 and terminates at 5450, and thus overlaps the M2-1 ORF. To create a mutant HMPV in which the M2-2 ORF is silenced, single nucleotide substitutions were introduced at positions 5236 and 5248, which removed the two potential translational start codons without changing the amino acid coding assignment in the overlapping M2-1 ORF (FIG. 18A). A third nucleotide substitution was made at position 5272, which introduced a translational stop codon into the M2-2 ORF. In addition, nucleotides 5289-5440 were deleted, which deleted most of the M2-2 ORF. These changes were introduced into the wild type rHMPV antigenomic cDNA as well as into the rHMPV-GFP antigenomic cDNA. In each case, the Δ M2-2 virus was recovered successfully from cDNA. The rHMPV Δ M2-2 and rHMPV-GFP Δ M2-2 viruses were compared to their respective parents, rHMPV and rHMPV-GFP, with regard to multi-step growth kinetics *in vitro* (FIG. 18B). This showed that each Δ M2-2 virus replicated somewhat less efficiently than its rHMPV-GFP parent at many of the time points, although by the end of the experiment at 13 hours, the titers were very similar. These mutations are of interest for vaccine

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design, since they do not greatly affect growth in vitro (which is necessary for vaccine manufacture) and are likely attenuating in vivo. They are evaluated in the hamster model noted in Fig. 17B and C, and described more completely in a subsequent Example below. Northern blot hybridization was then used to monitor the synthesis of intracellular RNA by the Δ M2-2 virus. As shown in FIG. 18C, the synthesis of genome and antigenome by the Δ M2-2 virus was not greatly different than that of wild type HMPV. However, the Δ M2-2 virus directed a level of mRNA synthesis that, when normalized to the amount of genome from replicate gel lanes, was up to 8.7-fold that of wild type HMPV. The up-regulation of protein synthesis without a concomitant increase in virus replication would be highly desirable for a vaccine virus. This phenotype resembles, but is not identical to, that of the Δ M2-2 mutant of human RSV. For RSV, the Δ M2-2 mutant exhibited a delay in the accumulation of mRNA that was not observed here, and exhibited a delay and reduction in the accumulation of genome and antigenome that also was not observed for HMPV. Further unanticipated differences between the HMPV and RSV are described herein, namely the ability to disrupt the HMPV M2-1 ORF, or to delete M2-1 and M2-2 altogether, without loss of viral viability for HMPV whereas either mutation was lethal for RSV.

In another application, illustrated in FIG. 19, a series of single amino acid substitutions was introduced into the cysteine-histidine motif present in the predicted M2-1 protein of rHMPV-GFP. This "Cys3-His1" motif consists of three cysteine residues at M2-1 amino acid positions 7, 15, and 21, and a histidine residue at position 25 (FIG. 19). This motif is exactly conserved in RSV and other pneumoviruses. In RSV, the integrity of this motif is essential for the transcriptional processivity and anti-termination function of M2-1, and is essential for the recovery of infectious recombinant virus (Hardy and Wertz *J. Virol.* 74:5880-5885, 2000; Tang *et al.*, *J. Virol.* 75:11328-11335, 2001). The amino acid substitutions introduced into the M2-1 protein of rHMPV-GFP were as follows: Cysteine-7-Serine (C7S), Cysteine-15-Serine (C15S), and Histidine-25-Serine (H25S). As controls, two additional mutants were made involving amino acids that are found in the vicinity of the motif but are not considered to be part of the motif: Tyrosine-9-Serine (Y6S) and Asparagine-25-Serine (N25S). Each of these mutants was readily recovered as infectious virus. The C7S, Y19S, C15S, N16S, H25S and Δ M2-1 mutants of rHMPV-GFP were evaluated for multi-cycle growth in LLC-MK2 cells in parallel with rHMPV and rHMPV-GFP, as shown in FIG. 20. The mutants exhibited a range of growth efficiency relative to the rHMPV-GFP parent, with the Y9S mutant replicating more efficiently, the C7S mutant replicating comparably, and the others replicating somewhat less efficiently. Nonetheless, with the exception of the H25S mutant, the final titers at 13 days were mostly comparable to that of the rHMPV-GFP parent.

In another mutation involving the M2-1 ORF of rHMPV-GFP, the ATG translational start site was changed to a TAG termination codon, as shown in FIG. 21, resulting in the mutant designated rHMPV-GFP Δ M2-1. The next ATG codon is located far down the ORF, at codon 134 out of 187 codons, and hence this mutation should ablate synthesis of M2-1. In this same mutant,

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additional termination codons were introduced closely downstream in each of the three reading frames to further preclude possible ribosomal entry and passage down the ORF. The HMPV-GFP- Δ M2-1 mutant was readily recovered as infectious virus.

5 The ability to rapidly and effectively recover this panel of mutants in the M2-1 ORF was a further demonstration that the cDNA-based recovery system is a robust method for expeditiously developing rHMPV variants bearing predetermined changes. The finding that a number of these mutants replicated more efficiently than their direct parent rHMPV-GFP suggests that such mutants would be valuable for use in immunogenic compositions, since improved replication *in vitro* would greatly facilitate the production of rHMPV and also might be an improved source of HMPV antigen for a protein-based immunogenic composition. In these embodiments, rHMPV-GFP was used as a parent because the expression of the GFP marker facilitates *in vitro* characterization. These forms also can be directly evaluated in experimental animals, and the presence of the marker would facilitate analysis of possible effects on virus localization *in vivo*. In addition, non-GFP versions can readily be generated, as was done above for the Δ SH, Δ G and Δ SH/G viruses, which would be appropriate for clinical evaluation. The finding that the Cys3His1 motif, and the M2-1 ORF altogether, are not required for efficient growth *in vitro* was completely unanticipated, particularly since with the Cys3-His1 motif and M2-1 protein were previously shown to be essential for the recovery of infectious RSV (Tang *et al.*, J. Virol. 75:11328-11335, 2001). In addition, it was independently confirmed that mutations in the Cys3-His1 motif of RSV, or silencing the M2-1 ORF of RSV, prevented the recovery of recombinant RSV. Although the Cys3-His1 motif is not required for efficient HMPV replication *in vitro*, it is reasonable to anticipate that this motif and this protein have been conserved in HMPV for functional reasons and do contribute to some aspect of HMPV replication or interaction with its host. Thus, it is reasonable to anticipate that some of these mutations will be found to be attenuating *in vivo* and useful for inclusion in a live attenuated HMPV immunogenic composition.

25 In another exemplary embodiment the HMPV M2 gene was deleted altogether from rHMPV-GFP, including its GS and GE signals and both the M2-1 and M2-2 ORFs (FIG. 22A). The resulting rHMPV-GFP- Δ M2(1+2) virus was readily recovered and propagated *in vitro*. The Δ M2-2, Δ M2-1, and Δ M2(1+2) viruses were compared with wild type HMPV with regard to the efficiency of multi-cycle replication in LLC-MK2 and Vero cells (FIG. 22B, upper and lower panels, respectively). This experiment was performed with versions of the viruses that express GFP in order to facilitate visualization of growth, but the expression of GFP was not otherwise relevant to the experiment. In LLC-MK2 cells, each of the M2 mutant viruses replicated more than 10-fold less efficiently than wild type. In contrast, in Vero cells each of the M2 deletion viruses grew to a final titer that equaled or exceeded that of wild type HMPV. One of the important differences between LLC-MK2 and Vero cells is that the latter lack the structural genes for type I interferon. Thus, this result suggested that that the M2 deletion viruses were more sensitive to interferon than wild type HMPV. In addition, the

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observation that the M2 deletion viruses replicated efficiently in Vero cells is important, since this is a cell substrate that is acceptable for preparing vaccines for humans.

To directly evaluate the interferon-sensitivity of the M2 deletion viruses, Vero cells were pre-treated overnight with a range of concentrations of type I interferon in order to induce an antiviral state. The ability of the Δ M2(1+2) and Δ M2-2 viruses to replicate was evaluated in the interferon-treated cells compared to wild type HMPV and wild type HRSV. Each of the viruses used in this experiment expressed GFP for the purpose of monitoring the infections, but the presence of GFP was not otherwise relevant to the results. Cell monolayers were infected at an moi of 1 PFU per cell (or a multiplicity of infection (moi) of 0.01 in the case of RSV, reflecting its more efficient growth) and incubated for 4 days. Control monolayers were mock-interferon-treated, infected and processed in parallel. The media supernatants were harvested and analyzed by plaque assay to determine virus titers. The fold reduction between each interferon-treated culture and its corresponding mock-interferon-treated control was calculated and is shown in FIG. 22C. Thus, the replication of wild type rHMPV-GFP were reduced from 5-fold to 1680-fold by increasing amounts of interferon, compared to mock-interferon-treated controls. In comparison, the Δ M2(1+2) and Δ M2-2 viruses were more sensitive and were reduced 19-fold and 13-fold, respectively at the lowest concentration and were completely inhibited at the highest concentration. Viruses that exhibit increased sensitivity to interferon typically are attenuated *in vivo*. Thus, these results suggest that these viruses are candidates to be attenuated derivatives.

Another exemplary embodiment of the current disclosure involves changing gene order to achieve attenuation, increase antigen expression, or achieve some other desirable phenotype. In a subsequent Example, the F protein of HMPV is identified as a major protective antigen, and the G and SH are also identified as virion surface proteins, which makes them likely protective antigens. In the wild type gene order, SH, G and F are located at gene positions 6, 7 and 4, respectively. It is anticipated that HMPV transcription will exhibit a polar gradient, in which promoter-proximal genes are expressed more efficiently than downstream ones. Thus, placement of putative protective antigen genes such as SH, G, and F proximal to the promoter, either singly, as a pair, or as a triplet, will result in more efficient expression. In this application, the genes can be moved to this position from their wild type location, such that the virus retains a single copy of each, or the genes can be inserted as a second copy, such that the virus now expresses two copies of one or more of the HMPV protective antigens. The change in gene expression due to the relocation of genes or to the addition of a second copy can have other desirable effects, such as increased or decreased growth *in vitro* or *in vivo*.

Exemplifying this strategy of "shifting" gene position for developing HMPV derivatives with improved properties, the position of the SH-G gene pair was altered in rHMPV-GFP (FIG. 23A). In one construct, designated "Order #1", the SH-G gene pair was moved from its position following the M2 gene to a position preceding the F gene. This results in a gene order in that region of the genome that mimics that of RSV, namely: M-SH-G-F-M2-L. Hence, the positions of four

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genes were altered in "Order 1", namely SH and G (each moved upstream by two positions) and F and M2 (each moved downstream by two positions). Accordingly, any gene or combination of genes can be moved within the genome, obtaining changes in gene expression that can provide desirable phenotypes including increased antigen expression, improved growth *in vitro*, and attenuation. As shown in FIG. 23B, the kinetics and magnitude of replication of the mutant called RSV Order #1 was indistinguishable from that of its direct parent rHMPV-GFP. This showed that changes in gene order can readily be achieved, resulting in infectious virus that replicates efficiently *in vitro* and can be evaluated for desirable properties such as attenuation or improved antigen expression *in vivo*.

As another permutation shown in FIG. 23A, the SH-G pair was moved to be upstream of the F gene in rHMPV-GFP, and a second copy of the pair was inserted, resulting in the construct "Order # 2". Thus, this construct contains two copies of the SH and G genes, which are potential protective antigens. In this case, the F-M2 gene pair has been moved a total of four positions downstream relative to the viral promoter, and the L gene is now two positions further downstream compared to the parental wild type HMPV. Yet another strategy of "gene shifting" involves moving the presumed protective antigen G and F genes from their normal positions as the 7th and 4th genes, respectively, in the HMPV gene map to promoter-proximal positions. Each gene can be moved individually or as a pair, as shown in FIG. 24A. In the case of the constructs shown in FIG. 24A, the promoter-proximal insertion site will be the same as was used for insertion of the GFP transcription cassette to make the rHMPV-GFP construct (FIG. 9), from which efficient expression has been demonstrated. While these represent preferred signals and insertion sites, other signals and insertion sites can readily be evaluated and used by the methods of this current disclosure. Preferred insertion sites would be ones in proposed intergenic regions or regions of genes that are outside of the ORFs and putative transcription signals and thus more likely to accommodate engineering without affecting HMPV replication. A subsequent Example provides identification of genome regions that are poorly conserved between divergent strains of HMPV, suggesting that they are less likely to be essential, which provides further guidance to identify preferred insertion sites. The transcription cassette should preferably be designed so that, following its insertion into the antigenomic cDNA clone, the inserted ORF is flanked by a set of GS and GE signals and each gene in the backbone similarly is flanked by a set of transcription signals. In the gene-shift constructs shown in FIG. 24A, insertion of the G and/or F gene into a promoter proximal position was accompanied by deletion of the corresponding copy from the normal antigenome position: in instances where G was shifted to the first or second position, nucleotides 6101-6960 were deleted to delete G from its natural location, and in instances where F was shifted, nucleotides 3053-4710 were deleted to delete the naturally-occurring copy of F. Analysis of the multi-step growth kinetics of these viruses *in vitro* (FIG. 24B) showed that each replicated somewhat less efficiently than the wild type parent, although the Final titers of the single-gene shift mutants (rHMPV-G1 and F1) were identical to that of the rHMPV parent, while the double-gene shift mutants were only slightly lower.

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A further set of exemplary viruses were constructed in which the G and F genes were inserted into the promoter proximal positions, but the copies of the genes in their natural positions were left undisturbed, as shown in FIG. 25A. Thus, rHMPV+G1 and +F1 contains two copies of the indicated gene (note that the symbol "+" in the designation indicates that the genes were added rather than "shifted"). Similarly, rHMPV+G1F2 and +F1G2 contained an additional copy each of G and F in the indicated position, and rHMPV+G1F2F3 contained an additional copy of G and two additional copies of F in the indicated positions. Interestingly, the multi-cycle growth kinetics of these viruses were somewhat reduced compared to their rHMPV parent (FIG. 25B), but among themselves the various addition mutations grew with similar kinetics. Thus, while the addition of one gene was somewhat attenuating, as had also been observed with GFP, the further addition of one or two more genes had little effect, even in the case of the relatively large F gene. The level of replication in this particular experiment had not plateaued, and the final virus yields were not determined. In these particular constructs, the added genes were of the same virus strain. However, it also should be feasible to incorporate one or more genes from a different strain, such as one possessing antigenic differences. Thus, for example, a rHMPV+G1F2 virus could be made that expresses F and G pairs representing two different strains, resulting in a single bivalent recombinant virus that would elicit a broader immune response.

The presence and intactness of the additional genes in the genome of recovered rHMPV+G1F23 were investigated by RT-PCR performed on total RNA from recovered virus following three passages in Vero cells. RT-PCR was performed with a forward primer hybridizing to the HMPV leader region and a reverse primer hybridizing to N sequence, which yielded a single, major band of approximately 4.1 kb, the appropriate size to contain the tandem G1F2F3 supernumerary genes (not shown). The sequence of the supernumerary G1F2F3 genes could not be determined directly by RT-PCR consensus sequencing of viral RNA because primers specific to G or F would prime on each of the two copies of G and three copies of F, precluding analysis of each gene individually. Therefore, the supernumerary genes were first amplified by RT-PCR in three separate, overlapping segments. The first RT-PCR segment used a forward (positive-sense) primer (which also served as the RT primer) from the leader region and a reverse primer from the F gene (840 nt downstream of the F GS signal): although this latter primer would prime on each copy of F, priming from the promoter-proximal copy (F2, numbered as in rHMPV+G1F23) would be the most efficient in combination with this forward primer, and yielded a ~1.6 kb fragment that contained the leader, the G1 gene, and about 840 bp of the F2 gene, and was purified by gel electrophoresis for sequence analysis. The second segment was amplified by RT-PCR with an RT/forward primer located about 670 nt downstream of the F GS, and a reverse primer located 554 nt downstream of the F GS: although the forward primer would prime in all three copies of F and the reverse primer in all three copies of F, priming in F2 and F3 would be the only combination to result in successful amplification of a PCR fragment (~1.5 kb) that contained the downstream 978 bp part of the F2 gene, and a 554 bp

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upstream part of F3, and was purified by gel electrophoresis for sequence analysis. The third fragment was generated with an RT/forward primer from the upstream end of F (14 nt downstream the F GS signal) and a reverse primer from the upstream end of N: the forward primer would prime in all three copies of F, but priming in F3 would be the most efficient and would yield a ~1.75 kb product that contained most of the F3 gene, and 120 bp of the N gene, and was purified by gel electrophoresis for sequence analysis. This provided an RT-PCR consensus sequence of the G1F2F3 supernumerary genes that was free of mutations, and all GS/GE signals and intergenic sequences were correct, indicating that these added genes were stably recovered in recombinant virus.

To determine the effect of the gene additions on viral gene expression, cells were mock-infected or infected with biologically-derived HMPV83, rHMPV, or rHMPV+G1F23, and total intracellular RNA was isolated three days later and subjected to Northern blot analysis with double stranded probes to the F, G or M genes (FIG. 25C). The F probe detected a major band of the appropriate size to be the 1644-nt F mRNA, as well as a fainter band of genome and antigenome, which in the case of rHMPV+G1F23 would be 4008 nt larger compared to HMPV83 or rHMPV due to the presence of the three added gene copies (FIG. 25C, lane 4). There were only trace amounts of readthrough mRNAs detected with the F probe. Phosphorimager analysis indicated that the amount of genome plus antigenome for rHMPV+G1F23 compared to HMPV83 and rHMPV was 0.9 and 0.5, respectively (corrected for the two extra copies of the F gene present in rHMPV+G1F23). In comparison, the relative amount of F mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV was 6 in each case. Normalized to the respective value of genome plus antigenome, this corresponded to a 6.6- to 12-fold higher level of F mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV.

Northern blot analysis with the G probe detected, for each of the three viruses, a band of the appropriate size to be the 711-nt G mRNA as well as bands corresponding to SH-G and M-SH-G readthrough mRNAs and a band corresponding to antigenome and genome RNAs (FIG. 25C, lanes 6, 7 and 8). Phosphorimager analysis indicated that the amount of genome plus antigenome for rHMPV+G1F23 compared to HMPV83 and rHMPV was 1.0 and 0.5, respectively (corrected for the extra copy of the G gene present in rHMPV+G1F23), values very similar to that observed with the F probe noted above. In comparison, the relative amount of G mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV was 14-fold and 15-fold, respectively. This would correspond to a 14- to 30-fold higher level of G mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV. Thus, the expression of the G and F genes by the rHMPV+G1F23 virus indeed was greatly increased.

Yet another strategy for designing improved immunogenic compositions against HMPV is to modify the ORFs encoding protective antigens so that the codon usage is consistent with efficient translation. Specifically, for many of the codons in a given ORF, the degeneracy of the genetic code allows for more than one choice of codon without changing the amino acid coding assignment. In a number of cases, specific codon choices have been shown to be associated with efficient translation while, conversely, other codon choices are associated with decreased efficiency of translation. Thus,

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the ORFs encoding HMPV antigens can be re-engineered to contain codon choices consistent with efficient translation. This can be done for ORFs in their natural genome positions as well as for ORFs that have been shifted or for heterologous ORFs that have been inserted.

Another exemplary embodiment of the current disclosure involves using a single attenuated
5 HMPV backbone to make immunogenic compositions against more than one HMPV strain, subgroup or serotype. In this application, the protective antigen genes of an attenuated derivative of HMPV, such as strain 83, are replaced singly or in toto by the corresponding genes of a heterologous HMPV. Provided that most of the attenuating mutations of the attenuated parent HMPV lie in genes other
10 than the protective antigen genes, this results in a chimeric virus in which the attenuated backbone of the parent bears the protective antigens of the heterologous strain. This chimeric virus can then be administered on its own, or can be combined with the original parent to make a two-virus bivalent immunogenic composition against the two viruses. This process can be repeated for additional heterologous strains as necessary.

Yet another exemplary embodiment of the current disclosure involves the addition of one or
15 more transcription cassettes encoding one or more foreign ORFs, as exemplified in Example II by GFP. In this application, the foreign gene is placed under the control of HMPV transcription signals and is inserted into the genome in a location that does not interfere with virus viability, such as an intergenic region or a gene noncoding region. In this way, a variety of foreign proteins can be expressed, such as ones encoding protective antigens of heterologous pathogens, or
20 immunomodulatory molecules.

In yet another exemplary embodiment, rHMPV-GFP was recombinantly modified to incorporate a Phenylalanine to Leucine mutation at amino acid position 456 in the L protein, a mutation designated F456L. This particular mutation was suggested by amino acid sequence alignment as corresponding to a position that encodes attenuating mutations in other, heterologous
25 mutant nonsegmented negative stranded RNA viruses. The sequence alignment in FIG. 26A shows a phenylalanine-521 to Leucine (F521L) substitution in the L protein specified by a mutation in the L gene of an RSV cold-passage temperature-sensitive (*cpts*) mutant *cpts*530 (Juhász *et al.*, J. Virol. 71:5814-9, 1999). The wild type F521 residue in the RSV sequence is exactly conserved among other mononegaviruses examined and, in particular, is present in HMPV. It should be noted that the amino
30 acid position of the corresponding residue is not the same in each L protein, reflecting differences in length as well as small deletions and insertions elsewhere in the various individual molecules. Thus, the corresponding residue is F456 in HMPV. From this comparative mapping analysis, the F456 residue in HMPV is identified as a target for either an identical, conservative, or even non-conservative amino acid substitution (for example, substitution of the F456 residue by a leucine, or
35 by a conservative or non-conservative amino acid as compared to leucine). In this particular Example, the F456L mutation was introduced into rHMPV-GFP and was readily recovered in a recombinant virus, designated rHMPV-GFP-F456L. As shown in FIG. 27, this mutant replicated *in vitro* with an

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efficiency that was indistinguishable from that of its direct parent, rHMPV-GFP, a characteristic that is important for efficient viral recovery, evaluation and manufacture. Thus, this mutant is an excellent candidate for further evaluation to determine its phenotypic characteristics *in vitro* and *in vivo*.

Further description of this general strategy, including discussion of FIGS. 26B-26F and 28, is provided above, along with other strategies for the expeditious design of attenuated derivatives based on the methods of this current disclosure.

Yet another strategy for making live attenuated vaccine candidates is the "Jennerian" approach, which involves the use of a mammalian or avian virus to immunize against an antigenically related human virus. This approach is named after Edward Jenner's successful use of cowpox as the initial vaccine against smallpox in humans. This approach is based on the idea that a virus that has evolved to replicate efficiently in its natural animal host often will replicate inefficiently and thus be attenuated in the non-natural human host, reflecting a host range restriction. Such a host range restriction typically is a very stable phenotype, being the aggregate effect of the many nucleotide and amino acid sequence differences between the animal and human viral counterpart. A phenotype that is based on many loci would be refractory to drift.

Other examples of "Jennerian" vaccines include the use of bovine rotavirus or bovine PIV as candidate vaccines against the respective human viruses. This can involve using the animal virus as it is provided by nature, as illustrated by the use of bovine PIV directly as a vaccine (Karron *et al.*, *Pediatr Infect Dis J* 15:650-654, 1996). Alternatively, the virus can be modified so that some of the genes are derived from the animal virus and some from the human virus. In such a case, it is preferable to have the major protective antigens be derived from the human virus, such that the immunity that is induced will be maximally effective against the human virus, and one or more other genes be derived from the animal virus such that they confer host range restriction. One such situation involves chimeras between human and bovine parainfluenza type3 viruses: in one case, the attenuated bovine backbone was modified so that the protective HN and F antigen genes were replaced by those of the human virus, thus combining the attenuated bovine viral backbone with the antigenic determinants of the human virus (Schmidt *et al.*, *J. Virol.* 74: 8922-8929, 2000), and in a second case the human virus was attenuated by replacing a single gene, that of the nucleocapsid N protein, with that of its bovine viral counterpart (Bailly *et al.*, *J. Virol.* 74:3188-3195, 2000). Surprisingly, each of these bovine/human chimeric viruses was satisfactorily attenuated and immunogenic in rodent and nonhuman primates, and both are being prepared for clinical trials.

FIG. 30A illustrates chimeric HMPV vaccine candidates that were constructed by individually replacing the N, P or M ORFs of HMPV with the corresponding ORFs from APV subtype C. At the amino acid level, the N, P and M genes of HMPV and APV-C are 90, 69, and 89% identical, respectively. For comparison, the N protein of bovine parainfluenza type 3 virus, whose substitution into the human PIV backbone provided a promising vaccine candidate, also was within this range, being 85% identical with its human viral counterpart. Furthermore, there are A, B and D

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subtypes of APV that are less closely related to HMPV, providing a source of additional replacements ORFs whose greater degree of divergence might provide a higher degree of attenuation.

FIG. 30 Panels B, C and D illustrate the construction of chimeric N (Part B), P (Part C), and M (Part D) genes. In each panel, the upper sequence is that of a cDNA containing the native N, P or M HMPV gene, and the bottom sequence of each panel is a cDNA in which the HMPV ORF has been replaced by its APV counterpart. Both the native HMPV and chimeric HMPV/APV cDNAs have been constructed to be flanked by restriction recognition sites for enzymes (*BbsI*, *BsmBI*, *BfuAI*, as indicated) that cleave on the inner side of each recognition sequence to yield 4-nt overhangs. Since the actual cut site of these particular enzymes are not sequence specific, the cut sites can be designed to have the native HMPV sequence. This makes it possible to ligate the N, P and M genes together in a single step, as in FIG. 30E. Any combination of HMPV and HMPV/APV genes can be ligated. For example, ligation of HMPV/APV N with HMPV P and HMPV M would yield a virus in which the N ORF alone was derived from APV. Single, double, or triple ORF replacements can readily be made. This represents one strategy for introducing attenuating host range mutations, namely a strategy in which the backbone is derived from HMPV and one or more individual ORFs from APV are introduced. An alternative strategy that also can be achieved by the methods of this current disclosure involves starting with a full length APV genome and replacing the putative protective antigen genes, namely G, F and SH, by their HMPV counterparts in whatever combination is desired. This would combine the antigenic determinants of HMPV with the attenuated backbone of APV.

Example 4

Analysis of Genetic Diversity Between Distinct Antigenic Subgroups of HMPV for Development of Improved Immunogenic Compositions

When van den Hoogen *et al.* first described the isolation of HMPV, they proposed that there was significant diversity among the various isolates (also called strains) and noted that “it is tempting to speculate that these subgroups of hMPV isolates represent different serotypes of hMPV” (Nat. Med. 7:719-24, 2001). “Serotype” means that there would be insignificant antigenic cross-reactivity between HMPV subgroups following a primary immunization, and would necessitate the development of a separate vaccine for each. Even if the HMPV subgroups do exhibit antigenic cross reactivity, it could be that sufficient antigenic diversity exists such that neutralization and protection across subgroups is significantly reduced compared to within a subgroup, and it might be necessary to have both such subgroups included in an effective immunogenic composition against HMPV. Sequence diversity also was reported by Peret *et al.*, albeit following analysis of a small segment of the genome, who proposed that there may be two “major groups or lineages” of HMPV (J. Infect. Dis. 185:1660-3, 2002). This also is supported by recent sequence analysis of the N, P, M and F genes of a number of HMPV isolates (Bastien *et al.*, Virus Res. 93:51-62, 2003). The extent of sequence and antigenic diversity among circulating HMPV strains is of considerable importance to

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epidemiology, viral detection, and development of immunogenic compositions. Strains 83 and 00-1 as described herein are members of one of the proposed subgroups, and therefore a third strain was analyzed (75) that represents the second subgroup. Comparison of the three strains provided a measure of the degree of genetic diversity between subgroups (strain 83 or 00-1 versus 75) or within a subgroup (strain 83 versus 75).

A complete consensus sequence (FIGS. 39A-39D) was determined for the genome of strain 75 using the general procedures described in Example I above for strain 83. In this analysis, the sequence of the genomic termini of strain 75 were determined by 3' and 5' RACE as described in Example, I. Whereas strain 83 had yielded an unambiguous sequence for its entirety, strain 75 yielded a sequence in which a number of positions in the SH and G genes contained a mixture of two assignments. This suggested that the isolate was a mixture of two or more viruses that likely reflected quasispecies variants, either present in the original clinical sample or arising during *in vitro* passage. Analysis of cloned RT-PCR products spanning the SH and G genes produced two closely related sets of sequence that likely represented two different viruses. To obtain a sequence that could be unambiguously attributed to a single replication-competent HMPV 75 virus, the isolate was subjected to biological cloning by plaque isolation. Eight independent plaque-purified preparations yielded identical consensus sequences in the region of SH and G that was consistent with that of one of the two groups of cloned cDNA sequences. This sequence appears to represent the major constituent and was taken to be HMPV 75.

The genome of strain 75 is 13,280 nucleotides in length, compared to 13,335 for strain 83 and an estimated length of 13,378 for strain 00-1. The gene maps of strains 83 and 75, showing the cross-subgroup comparison, are shown in FIG. 29. Overall, the two genomes shared 80% nucleotide identity, compared to 81% between the A2 and B1 strains of RSV representing the two RSV antigenic subgroups A and B, respectively (Genbank accession numbers M74568 and AF013254, respectively). Similarly, the nucleotide sequence of isolate 75 (FIG.s 39A-39D) was 80% identical to that of isolate 00-1 (excluding the regions for which sequence was unavailable for 00-1), representing a second cross-subgroup comparison. The sequences of isolate 83 (FIG.s 37A-37D) and 00-1, representing an intra-subgroup comparison, were 92% identical. As will be detailed further herein, the level of divergence between the two HMPV subgroups bears considerable similarity to that between the two antigenic subgroups of RSV. This finding was unpredicted and is of considerable value because there is extensive information on the significance of RSV diversity for epidemiology and development of immunogenic compositions, and this provides a very useful context for consideration of the divergence between HMPV subgroups, particularly since the overall magnitude is similar. As will be noted herein, there were some differences between the diversity of HMPV versus RSV, but even here RSV provides a useful benchmark for evaluating these differences.

For most genera of the Paramyxovirus family, there is a "rule of six" whereby the nucleotide length of each genome is an even multiple of six (Kolakofsky *et al.*, J. Virol. 72:891-899, 1998). In

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many cases, the placement of cis-acting signals relative to hexamer spacing also follows a conserved pattern. However, HMPV replication and gene expression do not appear to be ruled by a phasing requirement based on the following observations: (i) the sequences for the three available isolates differ in length, as mentioned above; (ii) no integer from 2 to 9 is evenly divisible into each of the three genomes, and (iii) there was no apparent pattern of phasing for the gene boundaries within or between isolates. The absence of a phasing requirement also is suggested by the ability to recover a number of recombinant viral mutants possessing a wide variety of lengths, as shown in previous Examples. The absence of such a requirement was unpredicted, and will greatly facilitate the construction of recombinant HMPVs.

The availability of complete genome sequences representing the two HMPV genetic subgroups provides an opportunity to examine the relatedness of the proposed HMPV transcription signals between subgroups. This is illustrated in FIG. 31, which compares the putative GS, GE and intergenic sequences between the two subgroups. The GS and GE signals exhibited extensive sequence identity among the difference genes and between the two subgroups, and a number of residues were exactly conserved in all sequences. In contrast, the intergenic regions exhibited only 48% identity between subgroups, and non-coding gene regions that were not part of the putative GS and GE signal exhibited only 54% identity. This sharp distinction in the pattern of sequence identity, combined with the functional analysis for transcription activity illustrated by the the GFP expression cassette in Example II above, provides guidance and an experimental method for a clear delineation of these important cis-acting sequences. Conversely, the identification of regions such as the intergenic regions and the non-translated, non-signal gene regions points out areas where the likelihood is increased that modifications involved in gene insertions, gene rearrangements and other genetic engineering would be tolerated, providing guidance that will facilitate production of recombinant viruses for use in immunogenic compositions.

The conserved sequence at the up-stream end of each gene, which would include the GS signal, consists of 16 nucleotides, consensus: GGGACAAnTmnAATG (FIG. 31). One unusual feature of the GS signal of all HMPV isolates sequenced to date is the presence of ATG at positions 14-16 (FIGS. 8 and 31), which initiates the major ORF. In the SH gene of strains 75 and 00-1, there is an additional upstream ATG at GS positions 8-10: however, this start site is unlikely to be used because of its unfavorable sequence context. The methods of the current disclosure allow for a determination to be made whether the ATG at positions 14-16 is a functional element of the HMPV GS signal or whether the conserved spacing of this translational start site plays some other role in gene expression. This can readily be accomplished using either complete recombinant virus or the minigenome system. The downstream end of each HMPV gene is delineated by the putative 12- to 15-nucleotide GE motif (consensus: AGTTAnnnAAAAA), consisting of (in positive sense) a highly conserved AGTTA pentamer, followed by a poorly conserved A/T-rich trinucleotide followed by a tract of 4 to 7 A residues. A sequence that resembles the GE motif also is present near the

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downstream end of the HMPV leader region (FIG. 31), and the methods of the current disclosure will clarify whether this functions like a GE signal.

FIG. 32 shows the percent nucleotide identity and amino acid sequence identity between the two HMPV subgroups (strain 83 versus 75) and within a subgroup (strain 83 versus 00-1) for the various individual ORFs and their encoded proteins. With regard to nucleotide sequence identity between the two subgroups, the HMPV ORFs shared 81-87% nucleotide identity except for SH and G, which were substantially more divergent (69% and 59% identity, respectively). This pattern resembled that of the two subgroups of HRSV, for which the percent nucleotide identity for the various ORFs was 81-85% identical except for the more divergent M2-2, SH and G ORFs (69%, 77%, and 67% identity, respectively; FIG. 32). As noted above, the intergenic regions and the noncoding gene regions (exclusive of GS and GE signals) were 48% and 54% identical, respectively, between subgroups, compared to values of 42% and approximately 50%, respectively, for RSV (Johnson *et al.*, J. Gen. Virol. 69:2901-2906, 1988). Thus, the coding sequence for HMPV G was only marginally more conserved than HMPV noncoding gene sequence in general (59% versus 54%). Extensive nucleotide sequence divergence for the ORFs encoding the SH protein and, in particular, the G protein also was observed between two strains of the same subgroup (strain 83 versus 00-1, FIG. 32).

FIG. 32 also shows the amino acid sequence relatedness of the various HMPV proteins between the two subgroups (strain 83 versus strain 75), and within a subgroup (strain 83 versus 00-1). With regard to the total proteome, the HMPV subgroups shared 90% amino acid sequence identity, compared to 88% identity between the RSV subgroups. With regard to individual proteins, the most conserved HMPV proteins were N, M, F, M2-1, and L ($\geq 94\%$ identity between subgroups), followed by P and M2-2 (85%-89%), followed by the divergent SH (59%) and G (37%) proteins. SH and G are the only ones for which the percent relatedness at the amino acid level was less than at the nucleotide level, suggesting that amino acid substitutions were preferentially retained.

The pattern of HMPV sequence diversity between subgroups has general similarities to that of RSV (FIG. 32). For RSV, the "internal" virion proteins and the F protein constituted a group of highly conserved species, while the M2-2, SH and G proteins were more divergent. Three differences between HMPV and RSV were particularly noteworthy: (i) the HMPV M2-2 protein was markedly more conserved (89% amino acid identity between subgroups) than its RSV counterpart (61%); (ii) the HMPV F protein also was more conserved than was the case for RSV (95% identity between subgroups versus 89%); and (iii) the HMPV SH and G proteins were markedly more divergent than their RSV counterparts (59% and 37% identity between subgroups, respectively, compared to 72% and 55%, respectively). FIG. 32 also compares the HMPV proteins within a subgroup (strain 83 versus 00-1). For most of the proteins, the percent amino acid identity was very high ($\leq 95\%$), with the SH and G proteins being more divergent (85% and 70% identity, respectively). The value of 70% sequence identity for G within an HMPV subgroup is much lower than the value of

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91% reported for RSV G from the analysis of seven subgroup B strains (Sullender *et al.*, J. Virol. 65:5425-5434, 1991). Thus, the HMPV SH protein and, in particular, the G protein were markedly more divergent within and between subgroups than was the case for their HRSV counterparts. This finding was unpredicted and is important because it indicates these antigens would be ones for which it will be important to have both subgroups represented in an immunogenic composition once they are identified as significant protective antigens. A test for evaluating whether or not an HMPV protein is a protective antigen is presented below.

It was also surprising to find that the HMPV M2-2 ORF and protein exhibited a high level of identity between the two putative subgroups, since M2-2 of RSV is poorly conserved (FIG. 32). In the case of RSV, the M2-2 protein was identified as a functional gene product by the observation that disruption of its ORF in infectious recombinant virus resulted in altered virus growth and RNA synthesis (Bermingham and Collins, Proc. Natl. Acad. Sci. USA 96:11259-11264, 1999; Jin *et al.*, J. Virol. 74:74-78, 2000). As noted above, rHMPV in which the M2-2 ORF has rendered nonfunctional remains viable *in vitro*, although its phenotype remains to be fully defined. The high level of sequence conservation for the M2-2 ORF between HMPV subgroups suggests that it encodes a significant product, the absence of which might prove to be attenuating and useful in immunogenic compositions. The RSV M2-2 ORF is expressed by a ribosomal stop-restart mechanism, whereby ribosomes that exit the M2-1 ORF apparently double back and initiate translation at the overlapping M2-2 ORF (Ahmadian *et al.*, EMBO J. 19:2681-2689). For the HMPV M2-2 ORF, there are two possible methionyl translational start sites located 38 and 50 nucleotides upstream of the translational termination codon of the M2-1 ORF. These two sites are exactly conserved among all three strains compared here, and are embedded in highly conserved sequence. This is another example where the availability of the cross-subgroup comparison is useful for identifying structural features that are of potential significance, and whose possible functional roles can be tested using complete recombinant virus or minigenomes by the methods of this current disclosure.

The HMPV F, SH and G proteins appear to correspond to the three RSV surface glycoproteins F, SH and G, although there was clear sequence relatedness between HMPV and HRSV counterparts only for F (33% amino acid identity). For RSV, G and F are the only significant neutralization antigens and are the major protective antigens. The HMPV F protein is identified as a neutralization and protective antigen of HMPV in an Example below. The status of the remaining HMPV proteins as potential neutralization and protective antigens can be determined by the same method. The RSV F protein has a high degree of antigenic relatedness between subgroups (Johnson *et al.*, J. Virol. 61:3163-3166), consistent with its sequence relatedness (FIG. 32), and is the major contributor to HRSV cross-subgroup neutralization and protection. For HMPV F, amino acid sequence identity between subgroups was even higher than for RSV, and it is reasonable to anticipate that this similarity will make a substantial contribution to cross-neutralization and cross-protection between the HMPV subgroups. Conversely, the high degree of divergence of the G protein between

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and within HMPV subgroups likely will compromise its contribution to cross-neutralization and cross-protection to an even greater degree than is the case for RSV.

FIG. 33 shows amino acid sequence alignments between and within a subgroup for the SH (FIG. 33A) and G (FIG. 33B) proteins. The HMPV SH protein, like its RSV counterpart, is predicted to be a type II glycoprotein that is inserted in the plasma membrane by a hydrophobic signal/anchor sequence located near its amino terminus (FIG. 33A, boxed sequence), with a cytoplasmic amino terminus and an extracellular carboxy terminus. The SH proteins of strains 75, 83, and 00-1 varied in length (177, 179, and 183 amino acids, respectively; FIG. 33A) and were considerably longer than their 64- or 65-amino acid counterparts in RSV. The predicted extracellular domain has 2-4 motifs for N-linked glycosylation, one of which is conserved in all three isolates, as well as 3 or 4 potential sites for O-glycosylation (Hansen *et al.*, Glycoconj. J. 15:115-130, 1998) that are clustered within residues 75-81 in all three isolates (FIG. 33A). In addition, the SH proteins of the three isolates contain 9 or 10 cysteine residues, which are mostly in the extracellular domain, 9 of which are conserved among all three strains. The differences in length and amino acid sequence between the SH proteins of the different isolates were concentrated in the extracellular domain.

The HMPV G protein (FIG. 33B) also is a type II surface protein, bearing a general resemblance (but no significant sequence relatedness) to the G protein of RSV. The amino acid lengths of the G proteins of strains 75, 83, and 00-1 were 236, 219, and 236, considerably shorter than their 289- to 299-amino acid counterparts in RSV. The HMPV G protein, like its RSV counterpart, contains a high percentage of serine plus threonine residues (32- 35% for the three isolates, compared to a data base average of 13%) and a somewhat elevated level of proline residues (7-8.5%, compared to an average of 5%). Strains 83, -75 and 00-1 had 1, 4 and 4, respectively, potential acceptor sites for N-linked glycosylation in the ectodomain. Each HMPV G protein contained more than 40 predicted acceptor sites for O-linked carbohydrate (not shown): by analogy to RSV it is likely that not all of these are used, and usage might be heterogeneous. The serine, threonine, proline residues and the predicted sites for O-linked sugars were concentrated in the predicted extracellular domain, suggesting that this region has a long, extended, heavily glycosylated "mucin-like" structure, as is thought to be the case for RSV.

The HMPV G protein also resembles that of RSV in having most of the amino acid divergence localized to the extracellular domain (FIG. 33B). Specifically, the cytoplasmic and transmembrane domains combined were 64% and 96% identical between and within subgroups, respectively, compared to the remarkably low values for the extracellular domain of 25% and 61% identity between and within subgroups. For comparison, the value of 25% amino acid sequence identity is comparable to that observed for the attachment hemagglutinin-neuraminidase protein of PIVs from different genera, such as Genus Paramyxovirus versus Genus Rubulavirus or Avulavirus, and is substantially lower than the value of 42-66% identity observed for the influenza A hemagglutinin glycoprotein between subtypes H1, H2 and H3.

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The HMPV G protein lacks several of the prominent features of its RSV counterpart. HMPV G lacks the conserved 13-amino acid domain in the RSV G extracellular domain that is exactly conserved among all RSV strains and is partially conserved in bovine RSV (Johnson *et al.*, Proc. Natl. Acad. Sci. USA 84:5625-5629, 1987; Teng and Collins, J. Virol. 76:6164-6171, 2002). Indeed, the HMPV G ectodomain did not contain more than 3 adjacent amino acids conserved among all three isolates. HMPV G also lacks the four conserved, closely-spaced cysteine residues in HRSV G that partially overlap the 13-amino acid conserved domain, form a cystine noose, and include a CX3C chemokine motif (Tripp *et al.*, Nat. Immunol. 2:732-738 2001). The three HMPV G proteins in FIG. 33B each contained only 1 or 2 cysteine residues, with the only conserved one being at the inner face of the cytoplasmic domain, representing a potential acceptor site for fatty acid acylation. HMPV G also lacks a counterpart to the second methionyl translational start codon in the HRSV G ORF, which gives rise to a secreted form and is conserved between HRSV subgroups (Roberts *et al.* J. Virol. 68:4538-4546, 1994). Only isolate CAN75 has an appropriately located potential counterpart (methionine-43), and it is the fourth rather than the second methionyl residue. Thus, comparison of sequence features whose presence or absence is consistent across subgroups helps guide the identification of structural features as possible targets for mutational analysis and construction of chimeric recombinant viruses for use in immunogenic compositions according to the methods of this current disclosure.

The high level of divergence in HMPV SH and G proteins is consistent with the idea that there is a preference for retaining amino acid changes in these proteins. It is particularly noteworthy that the divergence was concentrated in the extracellular domain of each protein (FIG. 33A, B). A codon-by-codon examination of the nucleotide coding sequences for the extracellular domains of F, G and SH ORFs indicated that 75% of the codons containing a single nucleotide change were associated with amino acid substitutions, compared to 50% for SH and 7% for F. Based on the findings herein, it is suggested that the extraordinary frequency of amino acid substitution per nucleotide substitution in HMPV G (and to a lesser extent SH) reflects two factors: (i) selective pressure for amino acid change, which might come from host immunity, and (ii) the ability of the protein to tolerate substitution, which might be due to its proposed extended, unfolded nature. In contrast, HMPV F appears to resemble HRSV F, which is one of the more highly conserved proteins despite its status as a major HRSV neutralization and protective antigen. This likely reflects functional and structural constraints on amino acid substitution in this folded, globular glycoprotein. The time scale of nucleotide and amino acid substitution for HMPV is not known, but it was noted that, for HRSV, it is difficult to detect antigenic drift and thus the differences probably reflect a slow accumulation over years. These findings indicate that diversity in SH and G is an important consideration for HMPV epidemiology, virus detection, and development of effective immunogenic compositions.

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In conclusion, the present Example defines the amount genetic diversity that exists between the two proposed HMPV genetic subgroups and shows that, overall, the diversity between subgroups is similar in magnitude to that observed between the well-characterized RSV antigenic subgroups, although certain specific differences were observed. By comparing the two divergent HMPV subgroups, the identification of transcription signal motifs was further substantiated. Conversely, the identification of poorly-conserved regions such as the non-signal, non-protein-coding gene regions and the intergenic regions provides guidance in selecting these sites as being likely to be amenable to genetic manipulation. Importantly, the F glycoprotein was shown to be highly conserved between the two subgroups, which highlights the usefulness of this surface protein for incorporation in recombinant HMPV and chimeric recombinant HMPV of the current disclosure for production of broadly cross-reactive immunogenic compositions. Conversely, the other two viral surface proteins, SH and G, were demonstrated to be highly divergent between the subgroups, and will therefore be employed within recombinant HMPV and chimeric recombinant HMPV to contribute to subgroup-specific, rather than cross-subgroup, immunity (although they will often be used in multivalent immunogenic compositions as described herein above). The diversity of the two genetic subgroups at the antigenic level is characterized herein, and the importance of the F protein as a major neutralization and protective antigen is demonstrated.

Example 5

20 **The Use of Experimental Animals to Evaluate HMPV Infectivity and Immunogenicity**

Generation of Hamster Polyclonal Anti-HMPV Antibodies to the 75 and 83 Strains

To demonstrate that HMPV is infectious in a rodent animal model and can elicit an immune response characterized by production of HMPV-specific antibodies in the model host, three groups of 8- to 10-week-old golden Syrian hamsters (*Mesocricetus auratus*) were infected intranasally (IN) with approximately 10^3 - 10^4 TCID₅₀ of: a mixture of HMPV strains 75 and 83, HMPV 75 alone, or HMPV 83 alone. As described in the previous Example, strains 75 and 83 (also referred to as CAN75 and CAN83, respectively) represent the two genetic subgroups of HMPV and thus provide an evaluation of the biological characteristics of viruses from each subgroup and, in particular, give a measurement of the antigenic diversity between subgroups. Serum samples were collected on day 28 or 44 post-immunization and examined for their ability to detect HMPV infected monkey kidney cells (LLC-MK2) by indirect immunofluorescence. LLC-MK2 cells grown in OptiMEM (Invitrogen GIBCO) on glass slides were infected with a mixture of HMPV strains 75 and 83, strain 75 alone, or strain 83 alone, and were incubated at 32°C in OptiMEM medium with 5 µg/ml trypsin. At approximately 70 hours post-infection the cells were fixed and permeabilized with 3% formaldehyde and 0.1% Triton-X100 in PBS, as described previously (Skiadopoulos and McBride, J. Virol. 70:1117-24, 1996). Indirect immunofluorescence was performed with pooled hamster sera from animals that had been inoculated with HMPV 75 + 83, and fluorescein (FITC)-conjugated goat anti-

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Syrian Hamster IgG antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) were used for indirect immunofluorescence. As a control, serum from an adult human seropositive for HMPV was also used to detect HMPV infected cells in a comparable indirect immunofluorescence assay. Immunofluorescence was detected and images were collected on a Leica TCS-SP2 (Leica
5 Microsystems, Mannheim, Germany). Images were processed using the Leica TCS-NT/SP software (version 1.6.587), Imaris 3.1.1 (Bitplane AG, Zurich Switzerland), and Adobe Photoshop 5.5 (Adobe systems). Both the human serum and the serum from hamsters infected with HMPV strains 75 and 83 were able to detect LLC-MK2 cells infected with either HMPV strain 75 or 83, demonstrating that IN- administered HMPV is infectious and immunogenic in hamsters. Sera from animals that received
10 strain 75 alone or strain 83 alone were further analyzed as described herein.

Generation of Rabbit Polyclonal Antibodies to HMPV Strains 75 and 83

Specific pathogen free rabbits (Harlan Sprague Dawley) were surgically implanted with sterilized plastic wiffle balls and were immunized by injection of approximately 10^5 TCID₅₀ of
15 HMPV strain 75 or 83 into the wiffle ball, as described previously (Clemons *et al.*, Lab. Anim. Sci. 42:307-11, 1992). Fluid present in the chambers was collected on day 29, and the animals received two boosts by injection of HMPV preparations that had been purified and concentrated by centrifugation in sucrose gradient following conventional procedures: the first boost was on day 29 and the second was on day 57. Fluid present in the chambers was collected again on day 71. These
20 sera were analyzed as described herein.

Virus Titration and Serum Antibody Infectivity Neutralization Assays

Viral titer was quantified by serial 10-fold dilutions of virus applied to LLC-MK2 monolayer cultures on 96-well plates that were then incubated for 7 days at 37°C in OptiMEM
25 medium with 5 µg/ml added trypsin, as described previously (Newman *et al.*, Virus Genes 24:77-92, 2002). Virus infected monolayers were detected by incubation with hamster polyclonal antiserum, which had been raised by IN infection with a mixture of strains 75 and 83 as described above, followed by a second antibody consisting of peroxidase-conjugated rabbit anti-Syrian hamster IgG. Bound antibody-antigen complexes were detected by immunoperoxidase staining achieved with ECL
30 chromogenic substrate.

The susceptibility of HMPV to neutralization by HMPV-specific antiserum was determined using an endpoint dilution neutralization assay performed with heat inactivated hamster, rabbit or chimpanzee sera. The hamster and rabbit sera were prepared as described above. The chimpanzee sera were from 31 caged animals, as described further herein. Briefly, replicate aliquots each
35 containing approximately 100-200 TCID₅₀ of HMPV 75 or 83 were mixed with aliquots from a dilution series prepared from a heat-inactivated serum sample and incubated at 37°C for 1 hr. The virus-antiserum mixtures were then transferred to LLC-MK2 monolayers in 96-well plates and the

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cells were incubated for 1 hour at 32°C. The monolayers were washed two times with OptiMEM to remove serum and were overlaid with OptiMEM supplemented with 5µg/ml added trypsin. The cells were then incubated for 7 days at 32°C and infected monolayers were detected by immunoperoxidase staining with the polyclonal hamster anti-HMPV 75+83 antiserum and peroxidase-conjugated rabbit
5 anti-Syrian hamster IgG and ECL chromogenic substrate, as described above. The neutralization titer is read as the highest dilution of antibody at which the cultures were 50% reduced for infection. This assay was used to determine the HMPV neutralization titer of serum from infected animals, and also was used to screen animals to determine whether they had previously been exposed to HMPV.

With the availability of the HMPV-GFP virus, the neutralization assay can now be
10 simplified. In this case, replicate aliquots of HMPV-GFP virus (approximately 100-200 TCID₅₀) would be mixed with aliquots of a dilution series of serum as above, incubated for 1 hour at 32°C, and transferred to LLC-MK2 cells. The cells would be washed to remove serum, overlaid with OptiMEM supplemented with 5µg/ml added trypsin, and incubated for 7 days at 32°C. Infected cells could be visualized directly by fluorescent microscopy. The neutralization endpoint would be
15 indicated by the appearance of green fluorescence, indicative of infection. Many of the details of this assay can be varied as necessary, such as the period of incubation of HMPV-GFP and antibody, the choice of cell substrate, and the period of incubation of the cells. In particular, the use of HMP-GFP means that the cells can be monitored by fluorescent microscopy to select an optimal time at which to read the assay. The efficiency of virus titration was compared by visualization of GFP versus
20 immunostaining and found to be identical. However, detection by GFP was easier because the progression of the virus infection can be monitored and the plates read at an optimal time. In contrast, immunostaining can only be done once, and the timing is determined by estimating cytopathology by eye, which is more qualitative and not as precise. A further advantage is that GFP can be read directly, whereas immunostaining is a multi-step, time-consuming process requiring antibodies and
25 development reagents. Furthermore, the washing steps need in immunostaining frequently result in damage to or removal of the cell monolayer, which can complicate or ruin the assay. Thus, using rHMPV-GFP as an indicator virus for antibody titration (or evaluation of antiviral agents) provides a better method for titration.

30 *Evaluation of the Antigenic Relatedness of Strains 83 and 75*

As described in the previous Example, it has been suggested that HMPV isolates can be segregated into two genetic subgroups (van den Hoogen *et al.*, Nat. Med. 7:719-24; Peret *et al.*, J. Infect. Dis. 185:1660-3, 2002). The previous Example described in detail the level of genetic
35 diversity between the two proposed subgroups. The present Example (and also Example VI) describes the amount of antigenic difference between the two subgroups. The extent of antigenic diversity among circulating HMPV strains is of considerable importance to epidemiology and for development of effective immunogenic compositions.

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The antigenic relatedness between strains 75 and 83 was investigated by a cross neutralization study. As described above, antiserum was raised in rabbits by parenteral immunization of gradient-purified HMPV 75 or 83 followed by a boost of the same material, with serum obtained following each step. In addition, as described above, hamsters were infected IN with strain 75 or 83 and sera were obtained. These individual sera were assayed for the ability to neutralize both strain 75 and 83. Thus, this would compare the ability of antibodies from an animal exposed to either strain to neutralize the infectivity of the homologous strain versus its ability to neutralize the heterologous strain. If extensive antigenic differences exist, each serum should be much less effective in neutralizing the heterologous strain compared to the homologous strain.

As shown in Table 1, hamsters were immunized by intranasal infection with strain 83 or 75 and the sera were collected on day 44 and evaluated for the ability to neutralize each strain *in vitro*. Of the eleven animals that were infected with strain 75, there was no consistent pattern in which the homologous 75 strain was neutralized more efficiently than the heterologous 83 strain: four animals neutralized the homologous strain more efficiently (up to 3.0 log₂ difference, with animal 2164 exhibiting the greatest difference), but one (animal 2168) showed no difference and the remainder neutralized the homologous strain less efficiently (up to 3.0 log₂ difference, animal 2174). Conversely, all ten animals infected with strain 83 yielded sera that neutralized the homologous 83 strain more efficiently than the heterologous 75 strain, with a difference ranging from 0.4 log₂ (animal 2203) to 3.4 log₂ (animal 2201). Thus, each of the HMPV strains representing the two heterologous subgroups induced antibodies that efficiently neutralized the homologous and heterologous viruses, although antibodies from animals infected with strain 83 appeared to be somewhat less effective in neutralizing the heterologous versus the homologous virus.

Also as shown in Table 1, rabbits were immunized with either strain of HMPV and then boosted with the same strain, and post-primary immunization and post-boost sera were collected and tested for the ability to neutralize the homologous and heterologous subgroup virus. The initial immunization of rabbits resulted in sera that had relatively low neutralizing titers against either strain of HMPV, with titers in the range of ≤ 4.3 log₂ to 8.4 log₂. With these primary sera, there was no consistent difference between the efficiency of neutralization of the homologous versus the heterologous strain. Following the boost, the titers were substantially higher, in the range of 6.9 log₂ to 11.4 log₂, and in most cases the neutralization titer against the homologous strain was greater than against the heterologous strain. However, the difference was relatively modest, ranging from either no difference to a difference of 2.6 log₂. For example, animal R030, which had been immunized and boosted with strain 75, had a titer of 11.4 log₂ against the homologous strain 75 and a titer of 9.9 log₂ against the heterologous strain 83, corresponding to a 1.5 log₂ difference. The other rabbit (R031) that was immunized and boosted with strain 75 yielded serum that neutralized the homologous strain 75 four times (2.0 log₂) more efficiently than the heterologous strain 83. In the converse situation, with animals (R032 and R033) that had been immunized and boosted with strain 83, the titers following

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the boost either showed no difference for the heterologous versus homologous strain (animal R03), or showed 0.5 log₂ higher homologous versus heterologous titer (animal R033). Thus, out of the eight post-immunization and post-boost rabbit sera, one had a lower homologous versus heterologous titer, three had no difference, and four had a greater homologous versus heterologous titer, with the greatest difference being 2.6 log₂. This indicates a trend towards greater homologous versus heterologous reactivity, but the inconsistent nature of the pattern and the low magnitude is consistent with the interpretation that the two strains exhibit a very high degree of cross-neutralization *in vitro*. Thus, the high degree of antigenic relatedness observed with post-infection hamster sera was confirmed with rabbit sera both upon initial immunization and following a boost.

The high degree of antigenic cross-reactivity observed *in vitro* indicates that at least one major HMPV neutralization antigen or major epitope is conserved between the HMPV subgroups. The genetic analysis presented in the previous Example showed that, of the three HMPV surface proteins F, SH and G, the F glycoprotein was highly conserved between subgroups. In contrast, the SH and G glycoproteins were highly variable and neither protein had no more than three contiguous conserved amino acid in the extracellular domain, suggesting that conserved antigenic sites would be rare or nonexistent. The HMPV neutralization and protective antigens had yet been identified previously. In the case of RSV, the F and G surface proteins are the only significant neutralization antigens and the major protective antigens (Connors *et al.*, J. Virol. 65:1634-1637 1991), and in the case of the human PIVs the two surface proteins F and HN also are the neutralization and major protective antigens. A subsequent Example (Example VI) will illustrate identification of a major HMPV neutralization and protective antigen effective in conferring protection against infection with either genetic subgroup.

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Table 1: Homologous and heterologous HMPV antibody responses in rodents following immunization with HMPV strain CAN75 or CAN83

Rodent species and number	Immunizing virus ^{a,b}	Serum-neutralizing antibody titer ^c (recip. log ₂) against:	
		CAN75	CAN83
Hamsters			
2163	CAN75	8.9	6.9
2164	CAN75	8.3	5.3
2165	CAN75	7.9	5.9
2169	CAN75	9.9	10.4
2170	CAN75	8.4	7.3
2166	CAN75	8.4	8.9
2167	CAN75	8.9	9.9
2168	CAN75	8.4	8.4
2173	CAN75	9.9	12.7
2174	CAN75	9.4	12.4
2175	CAN75	9.9	11.9
Hamsters			
2201	CAN83	7.3	10.7
2202	CAN83	8.4	11.3
2203	CAN83	10.9	11.3
2204	CAN83	7.9	10.3
2205	CAN83	7.9	10.3
2206	CAN83	8.9	11.7
2207	CAN83	7.9	10.3
2208	CAN83	8.9	11.7
2209	CAN83	9.9	12.3
2210	CAN83	8.9	10.3
Rabbits			
R030	CAN75	≤ 4.3 ^d	5.9 ^d
R030 (p.b.)	CAN75	11.4 ^e	9.9 ^e
R031	CAN75	≤ 4.3 ^d	≤ 4.3 ^d
R031 (p.b.)	CAN75	11.4 ^e	9.4 ^e
Rabbits			
R032	CAN83	≤ 4.3 ^d	6.9 ^d
R032 (p.b.)	CAN83	6.9 ^e	6.9 ^e
R033	CAN83	8.4 ^d	8.4 ^d
R033 (p.b.)	CAN83	8.4 ^e	8.9 ^e

a. Hamsters were inoculated intranasally with 10⁴ TCID₅₀ of the indicated strain of HMPV.

5 b. Rabbits were immunized parenterally with 10⁵ TCID₅₀ on day 0 with the indicated strain of HMPV, and were boosted on day 28 with 10⁷ TCID₅₀ of gradient-purified virus of the same strain of HMPV.

c. Hamster neutralizing antibody titer, day 44 post-immunization serum. All animals had pre-immunization neutralizing antibody titers of ≤1.0.

10 d. Rabbit neutralizing antibody titer, day 28 post-immunization. All animals had pre-immunization neutralizing antibody titers of ≤1.0.

e. Rabbit neutralizing antibody titer post-boost, day 70 following the initial immunization. All animals had pre-immunization neutralizing antibody titers of ≤1.0.

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p.b.: post-boost

Prevalence of HMPV Serum Antibodies in Chimpanzees

5 Thirty-one captive chimpanzees (*pan troglodytes*) were screened for the presence of serum neutralizing antibodies to HMPV strain 75 or 83, using an *in vitro* infectivity neutralization assay with HMPV strain 75 or strain 83 as the antigen, (Table 2). Nineteen animals (61%) were found to be seropositive for either one or both HMPV strains. Serum neutralizing antibody titers to HMPV strain 75 ranged from undetectable ($\leq 1.0 \log_2$) to $\geq 9.6 \log_2$. Serum neutralizing antibody titers to
10 HMPV strain 83 ranged from $\leq 1.0 \log_2$ to $8.6 \log_2$. Twelve animals (39%) did not have detectable HMPV-75 neutralizing titers: these 12 animals also were seronegative to strain 83. Four additional animals were seropositive to strain 75 but seronegative to strain 83. These four animals had very low neutralizing antibody titers ($2.6 - 4.0 \log_2$), and their specificity is provisional. The prevalence of HMPV antibodies in chimpanzees indicates that these animals can become readily infected with
15 HMPV in captivity, most likely from their human handlers. It also is possible, and indeed likely, that there is animal-to-animal transmission. The ability of these animals to be infected by contact from humans or other animals is a strong indication of permissiveness to HMPV. The finding that very few animals were seropositive for only one of the two strains suggests that these strains are highly related antigenically in primates and likely represent only a single HMPV serotype.

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Table 2: Prevalence of HMPV-neutralizing antibodies in chimpanzee sera

Sample number	Chimpanzee ID No.	Serum neutralization titer (recip. log ₂) to indicated HMPV strain ¹ :	
		75	83
1	99A005	6.6	4.6
2	A0A001	7.0	5.6
3	A0A002	5.6	4.0
4	A1A005	1.0	≤1.0
5	A1A007	≤1.0	≤1.0
6	A1A001	2.6	3.6
7	A1A002	5.6	3.6
8	A1A003	3.6	≤1.0
9	A1A006	≤1.0	≤1.0
10	A0A006	4.0	≤1.0
11	A0A007	4.6	3.6
12	A0A005	2.6	≤1.0
13	A0A009	3.6	≤1.0
14	AOA003	7.6	4.6
15	99A004	7.6	6.6
16	A2A001	≤1.0	≤1.0
17	A2A004	≤1.0	≤1.0
18	A2A006	≤1.0	≤1.0
19	A2A009	≤1.0	≤1.0
20	A2A011	6.0	4.6
21	A2A013	6.6	4.0
22	A2A014	6.6	5.6
23	98A008	8.6	6.0
24	99A001	8.0	5.0
25	99A002	8.0	6.6
26	99A003	9.6	8.6
27	A1A010	≤1.0	≤1.0
28	A1A012	≤1.0	≤1.0
29	A1A013	≤1.0	≤1.0
30	A2A007	≤1.0	≤1.0
31	A2A008	≤1.0	≤1.0
Control serum (pre) ²		≤1.0	≤1.0
Control serum (post) ³		8.3	8.3

1. Numbers in parentheses are the titers expressed as the reciprocal dilution log₂.

2. pre-immune serum from hamsters before inoculation with both HMPV 75 and 83.

3. Post-immunization serum obtained from hamsters following inoculation with both HMPV 75 and 83.

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HMPV Infection of Chimpanzees and Monkeys: Virus Replication, Immunogenicity and Effect of Previous Exposure to HMPV

The ability of HMPV strains 75 and 83 to infect chimpanzees identified as seropositive or seronegative by the HMPV neutralization assay described above was evaluated. As shown in Table 3, eight animals were organized into four groups (two animals per group): group 1 was seronegative for strain 75 and 83. Group 2 and group 4 were seropositive for both strains. Group 3 was seronegative for 83 and had low or no detectable neutralization titers to strain 75. Groups 1 and 2 were administered strain 75, and groups 3 and 4 were administered strain 83. The animals were inoculated intranasally (IN) and intratracheally (IT) with 1 ml per site of $1 \times 10^{5.2}$ TCID₅₀/ml of the indicated virus. Administration was by both the intranasal (IN) and intratracheal (IT) routes to provide the opportunity for the virus to replicate at each site (Hall *et al.*, Virus Res. 22:173-84, 1992; Crow *et al.*, Vaccine 12:783-790, 1994). Animals were housed and specimens collected as previously described (Durbin *et al.*, J. Infect. Dis. 179:1345-51, 1999; Skiadopoulos *et al.*, Virology 260:125-35, 1999). The animals were observed clinically for 16 days post-inoculation. Nasopharyngeal swabs were collected on days 1 to 10 and 12 post-infection and tracheal lavages were collected on days 2, 4, 6, 8, 10, and 12 post-infection. Each of the four seronegative animals (groups 1 and 3) had nasal discharge on days 7, 8, 9 and 10 post-infection. As a precautionary measure, groups 1 and 3 were started on penicillin on day 8, because of evidence of congestion and noise in the lungs. One of the animals in group 1 had thick mucus in the day 8 post-infection TL sample and had decreased appetite. The seropositive animals (groups 2 and 4) showed no signs of illness throughout the course of the study. Thus, HMPV seronegative chimpanzees can develop illness following infection with the 75 and 83 HMPV strains demonstrating that these viruses are infectious and virulent in chimpanzees. Since the recombinant HMPV strain 83 virus differs from its biologically-derived parent by only four nucleotide differences involved in creating the *NheI* marker in the M/F intergenic region (FIG. 7), it is also considered a "wild type" virus in terms of genomic and phenotypic characteristics. Importantly, animals that were seropositive to both strains were protected from disease following challenge with either strain. This appears to be the first observation demonstrating the feasibility of immunizing against HMPV: since natural exposure protects against disease, it is reasonable to expect immunization with an appropriate immunogenic composition comprising a recombinant HMPV of the current disclosure will elicit a desired immune response as well. These results indicate that serum neutralizing antibody titers of 4.6 (animal AOA003) to 7.0 (animal AOA001) log₁₀ are sufficient to confer protection from HMPV disease. Results obtained with chimpanzees are particularly relevant given the close evolutionary relationship between chimpanzees and humans.

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Table 3: Infection of chimpanzees with HMPV

Group	Chimpanzee ID No.	Serum neutralization titer to HMPV strain:		HMPV administered ¹	clinical signs (day post-infection)	
		75	83		rhinorrhea	cough
1	A1A005	≤1.0	≤1.0	75	6,7,8,9,10,12	
	A1A007	≤1.0	≤1.0		7,8,9,10	
2	99A005	6.6	4.6	75		
	A0A001	7.0	5.6			
3	A1A006	≤1.0	≤1.0	83	7,8,9,10	
	A0A005	2.6	≤1.0		7,8,9,10	5
4	AOA003	7.6	4.6	83		
	99A004	7.6	6.6			

1. The animals were inoculated intranasally (IN) and intratracheally (IT) with 1 ml per site of 10^{5.2} TCID₅₀/ml of HMPV strain 75 or strain 83.

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The virus titers in the nasopharyngeal swabs and tracheal lavages taken from the HMPV-infected chimpanzees described in Table 3 were determined by an end point dilution assay with viral infectivity detected by immunostaining. As shown in Table 4, the chimpanzees shed relatively low levels of virus. This was particularly noteworthy for the animals in group 1, which were seronegative for both strains, and group 3, which was seronegative for strain 83: animals even though all of the animals in these groups exhibited clinical disease signs in response to infection, the mean peak titers of virus recovered from the nasopharyngeal swabs ranged from 1.9 to 3.2 log₁₀, and the titers from the tracheal lavage were 1.8 to 2.0 log₁₀. The swabs and lavages from animals in groups 2 and 4, which were seropositive to HMPV, either lacked detectable HMPV or had a very low titer (1.5 log₁₀). Thus, the lack of disease signs correlated with a reduced or undetectable level of virus shedding. The apparent incongruity between the low level of HMPV shedding in the presence of disease symptoms might reflect difficulty in retrieving infectious virus from secretions, possibly due to inactivation by host immune factors or a high level of cell-association of HMPV in the epithelial cells lining the respiratory tract of the chimpanzees. Since the chimpanzee is so closely related to humans, this raises the possibility that detection of HMPV in human secretions might also be inefficient. The inability to detect substantial HMPV shedding was unexpected, since RSV is known to be labile and cell-associated *in vitro*, but nonetheless is shed from RSV infected chimpanzees presenting with RSV-associated illness at a level that is 100 to 1000-fold greater than was the case for HMPV-infected chimpanzees (Belshe *et al.*, J. Med. Virol. 1:157-162, 1977). Alternatively, the level of HMPV replication in chimpanzees may indeed be low, but infection may trigger immune- or chemokine-mediated pathology resulting in development of disease symptoms (Glass *et al.*, Curr. Opin. Allergy Clin. Immunol. 3:467-473).

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Serum samples taken four to six weeks post infection contained high levels of serum neutralizing antibodies for every experimental group, with no clear difference between animals that had been seronegative or seropositive prior to the experimental infection. The post-immunization titers ranged from 7.4 to 10.2 log₁₀, considerably higher than the range of 4.6 to 7.0 log₁₀ that was associated with protection from clinical disease. Interesting, in this small sample there was no evidence of antigenic differences between strains 75 and 83. Specifically, seronegative animals that were immunized with either strain (groups 1 and 3) developed antibodies with titers that were somewhat higher against strain 83 than against strain 83, irrespective of the immunizing virus.

The animals that were seronegative for the strain used in the primary infection were next challenged with the heterologous HMPV strain. As shown in Table 4, both groups of animals were completely protected from cross-challenge, indicating that previous infection by one HMPV lineage protects chimpanzees from subsequent infection by the heterologous lineage. Thus, in the experimental animal that most closely resembles the human, immunity induced by an HMPV strain representing either genetic subgroup protected against infection with an HMPV strain from the heterologous subgroup.

It also was of interest to investigate the replication and immunogenicity of HMPV 75 and 83 in monkeys, which are more plentiful, less expensive and easier to house than chimpanzees. Twenty-two rhesus macaques and twenty African green monkeys were screened for serum neutralizing antibodies to HMPV, and each animal was seronegative to HMPV (≤ 1.0 log₂). This limited sampling suggested that these two species of monkeys are not readily infected with HMPV by exposure in captivity. Four animals of each species were infected and intratracheally with 10^{5.2} TCID₅₀ of either HMPV strain CAN75 or CAN83. Four animals of each species were infected and intratracheally with 10^{5.2} TCID₅₀ of either HMPV strain CAN75 or CAN83 at each site, and nasopharyngeal swab samples were taken at days 1 to 10 and 12 post-infection, and tracheal lavage samples were taken at days 2, 4, 6, 8, 10 and 12 post-infection (Table 5). The samples were assayed for virus titer by end point dilution. Illness did not develop in any of the monkeys. The infected rhesus monkeys had very low titers of virus, ranging from 0.9 to 2.6 log₁₀ TCID₅₀ (Table 5). Furthermore, the post-immunization titers of serum neutralizing antibodies were also relatively low, ranging from 6.3 to 8.4 log₂. Thus, rhesus monkeys support the replication of HMPV, but the level of replication is low.

Infected African green monkeys (Table 5) shed titers of HMPV ranging from 2.2 to 4.9 log₁₀ TCID₅₀, and developed serum neutralizing antibody titers that ranged from 9.4 to 10.9 log₂. The time course of HMPV replication in samples collected from the nasopharynx and the trachea of the African green monkeys is shown in FIG. 35, and indicates that virus shedding occurred over a broad window of more than six days. These data showed that African green monkeys are permissive for HMPV replication and, in particular, represent a suitable primate host in which to evaluate the replication and immunogenicity of rHMPV derivatives for use in immunogenic compositions. In particular, the highest titers of virus and antibody were obtained with strain 83, which is the one

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represented by recombinant HMPV of this current disclosure. In this regard, the ability to monitor HMPV replication up to almost 5 log₁₀ with concomitant immunogenicity provides a wide window for assessing effects on replication and immunogenicity due to the incorporation of attenuating mutations.

5 Thirty days following the first infection, the African green monkeys were challenged with the heterologous HMPV strain (Table 5). Both groups of animals were completely protected from cross-challenge, indicating that previous infection by one HMPV lineage confers resistance to subsequent infection by the heterologous strain. These data indicate that African green monkeys represent a suitable primate host in which to evaluate the protective efficacy of HMPV immunogenic compositions.

10 The studies described above provide data on the primary antibody response to strain 75 or 83 in seronegative animals, and describe the ability of the resulting antibodies to neutralize the homologous versus heterologous subgroup virus. These data were analyzed statistically by the Archetti-Horsfall formula (Archetti and Horsfall, J. Exp. Med. 92:441-462, 1950) to determine the
15 degree of antigenic relatedness based on *in vitro* neutralization. Each species was calculated separately, showing that the percent relatedness of the two HMPV subgroups was 48%, 64% and 99%, respectively, based on the data from hamsters, Rhesus monkeys and African green monkeys, respectively. This compares with a value of 25% and 31% for the two subgroups of RSV, based on analysis of animal and human sera, respectively (Johnson *et al.* J. Virol. 61:3163-3166, 1987; Hendry
20 *et al.*, J. Infect. Dis. 157:640-647, 1988). These data indicate that the two HMPV genetic subgroups are highly related antigenically and do not represent distinct serotypes or, likely, not even significant antigenic subgroups. This high degree of relatedness for the two HMPV subgroups is based on *in vitro* neutralization, and thus needs to be taken with some caution, since cross-neutralization *in vitro* might be mediated by a single highly conserved antigen, whereas cross-protection *in vivo* might
25 require the contribution of more than a single antigen, or might be augmented by the inclusion of additional antigens. Nonetheless, the present findings provide the first indication that the genetic diversity of HMPV can readily be accommodated by an HMPV vaccine, and that such a vaccine might need to represent only a single HMPV strain.

TABLE 4: Level Of Replication, Immunogenicity And Cross-Protection Of HMPV Strains CAN75 And CAN83 In Chimpanzees

Group no. ^a	Virus administered	Serostatus at time of inoculation	Pre-immunization serum neutralization titer ^d to:		Level of virus replication ^e in:		Post-immunization (day 28) serum neutralization titer ^d to:		Level of challenge virus replication ^f in	
			CAN75	CAN83	NP swab	TL	CAN75	CAN83	NP swab	TL
1	CAN75	negative ^b	$\leq 1.0 \pm 0.0$	$\leq 1.0 \pm 0.0$	1.9 ± 0.4	2.0 ± 0.8	8.7 ± 0.3	10.2 ± 0.3	$\leq 0.5 \pm 0.0$	$\leq 0.5 \pm 0.0$
2	CAN83	negative ^b	3.8 ± 1.5	$\leq 1.0 \pm 0.0$	3.2 ± 0.5	1.8 ± 0.3	8.9 ± 1.0	10.2 ± 0.3	$\leq 0.5 \pm 0.0$	$\leq 0.5 \pm 0.0$
3	CAN75	positive ^c	6.8 ± 0.2	5.1 ± 0.5	1.5 ± 0.0	$\leq 0.5 \pm 0.0$	7.4 ± 0.5	9.2 ± 0.2	nd	nd
4	CAN83	positive ^c	7.6 ± 0.0	5.6 ± 1.0	$\leq 0.5 \pm 0.0$	1.5 ± 0.3	8.4 ± 1.5	10.2 ± 0.2	nd	nd

a. Animals in groups of two were inoculated IN and intratracheally (IT) with $10^{3.2}$ TCID₅₀ of the indicated virus in a 1 ml inoculum at each site.

b. Chimpanzees in this group were seronegative (mean antibody titer of $< 1:4$) for the homologous infecting HMPV strain at the start of the study.

c. Chimpanzees in this group were seropositive (mean antibody titer of $> 1:20$) for the homologous infecting HMPV strain at the start of the study.

d. Serum neutralization antibody titer to the indicated virus is expressed as the mean reciprocal $\log_2 \pm$ standard error.

e. Level of virus replication is expressed as the mean of the peak virus titers (\log_{10} TCID₅₀/ml \pm standard error) for the animals in each group irrespective of sampling day. The NP titers on day 0 were $\leq 0.5 \log_{10}$ TCID₅₀/ml. The lower limit of detection is $1.0 \log_{10}$ TCID₅₀/ml. A value of $\leq 0.5 \log_{10}$ TCID₅₀/ml is assigned to samples with no detectable virus.

f. On day 35, animals were challenged IN and IT with $10^{5.2}$ TCID₅₀ of the heterologous strain of HMPV in a 1 ml inoculum at each site. Level of virus replication is expressed as the mean of the peak virus titers (\log_{10} TCID₅₀/ml \pm standard error) for the animals in each group irrespective of sampling day. The NP titers on day 0 were $\leq 0.5 \log_{10}$ TCID₅₀/ml. The lower limit of detection is $1.0 \log_{10}$ TCID₅₀/ml.

nd: not done

TABLE 5: Level Of Replication, Immunogenicity And Cross-Protection Of HMPV Strains CAN75 And CAN83 In Two Species Of Monkeys

Primate species	No. of animals	Virus inoculum ^a :	Level of virus replication ^b in:		Post-immunization (day 28) serum neutralization titer ^c to:	Percent antigenic relatedness ^d	Level of challenge virus replication ^e in:	
			NP swab	TL			NP swab	TL
Rhesus	4	CAN75	1.3 ± 0.1	0.9 ± 0.2	6.4 ± 0.6	64	nd	nd
Rhesus	4	CAN83	1.6 ± 0.1	2.6 ± 0.3	6.3 ± 0.1		nd	nd
African Green	4	CAN75	2.6 ± 0.5	3.2 ± 0.4	9.4 ± 0.3	99	1.4 ± 0.1	0.8 ± 0.1
African Green	4	CAN83	2.2 ± 0.2	4.9 ± 0.1	9.5 ± 0.5		0.6 ± 0.1	0.9 ± 0.2

a. Animals were inoculated IN and intratracheally (IT) with $10^{3.2}$ TCID₅₀ of the indicated virus in a 1 ml inoculum at each site.

b. The level of virus replication is expressed as the mean of the peak virus titers (\log_{10} TCID₅₀/ml ± standard error) for the animals in each group irrespective of sampling day. Virus titrations were performed on LLC-MK2 cells. The lower limit of detection is $1.0 \log_{10}$ TCID₅₀/ml. A value of $\leq 0.5 \log_{10}$ TCID₅₀/ml is assigned to samples with no detectable virus.

c. Mean serum neutralization antibody titer to the indicated HMPV strain is expressed as the mean reciprocal \log_2 ± standard error. All animals were seronegative for both strains of HMPV at the start of the study (that is, day 0 reciprocal neutralization titer of $\leq 1.0 \log_2$).

d. Percent antigenic relatedness was calculated using the formula of Archetti and Horsfall.

e. On day 30, animals were challenged IN and IT with $10^{5.2}$ TCID₅₀ of the heterologous virus in a 1 ml inoculum at each site. Level of virus replication is expressed as the mean of the peak virus titers (\log_{10} TCID₅₀/ml ± standard error) for the animals in each group irrespective of sampling day. The lower limit of detection is $1.0 \log_{10}$ TCID₅₀/ml.

nd: not done

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Development of a small animal model of HMPV replication

The evaluation of HMPV and rHMPV derivatives is further facilitated by the availability of a small animal model for evaluating replication and immunogenicity *in vivo*. Hamsters were infected intranasally with 10^6 TCID₅₀ of either strain 83 or 75, and animals were sacrificed on days 3, 4, 5, 6 or 7 and the nasal turbinates and lungs were harvested and assayed for virus titer. As shown in Table 6, each virus replicated to a peak titer greater than $6 \log_{10}$ in the nasal turbinates and 3.6 to $4.4 \log_{10}$ in the lower respiratory tract. Thus, hamsters were permissive for both subgroup strains. As shown in Table 1 (and in Table 7), infection with strain 83 induced serum neutralizing antibody titers of 10.3 to $12.3 \log_{10}$ against the homologous strain, and infection with strain 75 induced titers of 7.9 to $9.9 \log_{10}$ against the homologous strain. The basis for the apparent lower immunogenicity of strain 75 is not known, although this finding will need to be confirmed in a further study in which virus replication and immunogenicity are measured for each strain in the same study. The present data show that hamsters are relatively permissive for HMPV replication and provide a convenient small animal model for evaluating HMPV replication and immunogenicity.

The hamster model was used to evaluate cross-protection between the two HMPV genetic subgroups, as had also been investigated in chimpanzees as shown in Table 4. Groups of hamsters were infected with either strain of HMPV or with L15 diluent as a negative control. After 6 weeks the hamsters were challenged with either strain of HMPV and the level of pulmonary challenge virus replication in the respiratory tract on day 4 was determined, as described above. As shown in Table 7, previous infection by either HMPV strain induced a high level of protection against the homologous and heterologous HMPV strain in the upper respiratory tract. The lower respiratory tract was completely protected from challenge with either strain. In addition, sera were collected 31 days following the first infection and assayed to determine the neutralizing titer against each strain (Table 7). The results were very similar to those shown previously in Table 1: infection with CAN83 induced antibodies that were modestly more effective in neutralizing the homologous versus heterologous strain, but antibodies induced by infection with CAN75 neutralized the homologous and heterologous strains with similar efficiencies. These cross-protection data and reciprocal-neutralization assays indicated that CAN83 and CAN75 are antigenically highly related.

TABLE 6: DAILY LEVEL OF REPLICATION OF HMPV STRAINS CAN75 AND CAN83 IN THE UPPER AND LOWER RESPIRATORY TRACT OF INFECTED HAMSTERS

Virus inoculum ^a	Tissue	Mean virus titer (\log_{10} TCID ₅₀ /g \pm S.E.) on day ^b :				
		3	4	5	6	7
HMPV-CAN83	Nasal turbinates	6.0 \pm 0.5	5.7 \pm 0.3	5.1 \pm 0.3	3.7 \pm 1.2	1.7 \pm 0.5
	Lungs	3.6 \pm 1.0	3.1 \pm 0.3	3.0 \pm 0.3	\leq 1.5 \pm 0.0	\leq 1.5 \pm 0.0
HMPV-CAN75	Nasal turbinates	6.6 \pm 0.4	6.4 \pm 0.4	6.4 \pm 0.6	5.2 \pm 0.5	4.2 \pm 0.7
	Lungs	4.4 \pm 0.6	4.3 \pm 0.7	3.3 \pm 0.2	\leq 1.5 \pm 0.0	\leq 1.5 \pm 0.0

^aHamsters in groups of 30 animals were inoculated intranasally with 10⁶ TCID₅₀ of the indicated virus.

^bNasal turbinates and lung tissues from six animals from each group where harvested on days 3 through 7 post-infection. Virus present in tissue homogenates was quantified by serial dilution on LLC-MK2 monolayers at 32°C. Infected cultures were detected with hamster anti-HMPV antibodies and immunoperoxidase staining. Mean virus titer for each group of hamsters is expressed as \log_{10} TCID₅₀/gram \pm standard error, S.E.

TABLE 7: Hamsters Infected With HMPV Strain CAN83 Or CAN75 Are Protected Against Challenge With The Homologous And Heterologous HMPV Strains

Animals immunized with ^a	Mean serum-neutralizing antibody titer ^b (reciprocal log ₂) against:		Mean virus titer (log ₁₀ TCID ₅₀ /g) in nasal turbinates of hamsters administered the indicated challenge virus ^c :	
	CAN75	CAN83	CAN75	CAN83
CAN83	9.2 ± 0.2	10.4 ± 0.3	2.0 ± 0.2	1.7 ± 0.2
CAN75	10.9 ± 0.2	7.7 ± 0.2	≤1.5 ± 0.0	2.4 ± 0.5
L15 medium	≤1.0 ± 0.0	≤1.0 ± 0.0	5.3 ± 0.1	5.6 ± 0.2

a. Hamsters in groups of 12 were immunized by IN infection with 10^{3.5} TCID₅₀ of the indicated virus or mock-infected with L15 medium.

b. Sera were collected 2-3 days before and 31 days following the first infection, and the neutralizing titer against each of the two strains was determined.

The pre-infection anti-HMPV serum titers were ≤1.0 (reciprocal log₂) for all animals in the study.

c. On day 42, six hamsters from each group were challenged IN with 10^{5.5} TCID₅₀ of CAN83 and the remaining six were challenged with CAN75. Nasal turbinates and lungs were harvested four days later, and the virus titer was determined on LLC-MK2 cells. The mean virus titer for each group of hamsters is expressed as log₁₀ TCID₅₀/gram of nasal turbinate ± standard error.

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Example 6**Construction of a Human Parainfluenza Type I Virus (HPIV1) that Induces Neutralizing Antibodies and Protective Efficacy Against Both HMPV Genetic Subgroups**

To examine the ability of the HMPV F protein alone to induce serum neutralizing antibodies and to confer protection against HMPV challenge, a recombinant HPIV1 expressing the CAN83 F protein (rHPIV1-F₈₃) was recovered *in vitro* from an HPIV1 antigenomic cDNA containing the CAN83 F protein ORF inserted upstream of the HPIV1 N gene and flanked by HPIV1 GS and GE transcription signals (FIG. 36A). Expression of the F protein was confirmed by indirect immunofluorescence of infected cells (FIG. 36 B). Thus, rHPIV1-F₈₃ would express all of the proteins of HPIV1 as well as the F protein of HMPV.

Groups of hamsters were immunized with CAN83, wild type rHPIV1, rHPIV1-F₈₃ or with L15 medium, and 33 days later sera were collected and tested for the ability to neutralize the CAN75 and CAN83 strains. As shown in Table 8, sera from animals immunized with CAN83 or rHPIV1-F₈₃ efficiently neutralized both strains of virus. In each case, neutralization of the homologous CAN83 strain was somewhat more efficient than for the heterologous CAN75 strain. The mean neutralization titer of sera from rHPIV1-F₈₃-infected animals was 1.2 log₂ lower than that observed from CAN83-infected animals, which corresponds well with the lower titer of rHPIV1 replication in hamsters in this experiment compared to CAN83. Thus, when adjusted for the level of virus replication, the rHPIV1-F₈₃ vector, expressing only a single CAN83 antigen, was as efficient in inducing CAN83-neutralizing antibodies as CAN83 itself.

Fifty days following immunization, the animals were challenged by intranasal infection with rHPIV1, CAN75 or CAN83. As shown in Table 8, animals previously infected with rHPIV1-F₈₃ had a 125-fold and 158-fold reduction in the upper respiratory tract in the level of replication of the CAN75 and CAN83 challenge virus, respectively. Animals were completely protected in the lower respiratory tract. The rHPIV1-F₈₃ virus also protected against HPIV1 challenge, indicating that this virus was able to protect against the three respiratory tract pathogens, namely CAN75, CAN83 and HPIV1. This method also can now be used to determine the relative contribution of each of the other HMPV proteins to the induction of neutralizing antibodies and resistance to challenge virus replication. Importantly, these findings identify the F protein of HMPV as a major neutralization and protective antigen. Furthermore, the F protein of the CAN83 strain induced neutralizing antibodies and protection that were effective against both CAN83 and CAN75. This highlights the importance of this specific antigen in developing vaccines effective against all strains of HMPV.

TABLE 8: A Recombinant HPIV1 Expressing The CAN83 F Protein (RHPIV1-F₈₃) Equally Protects Hamsters Against Challenge With HMPV Subgroup CAN75 And CAN83 As Well As Against HPIV1

Virus inoculum ^a	Mean serum neutralization titer (log ₂) to indicated virus ^b :			Mean level of replication of the indicated challenge virus in the nasal turbinates ^c		
	rHPIV1	CAN75	CAN83	rHPIV1	CAN75	CAN83
L15 medium	≤1.0 ± 0.0	≤1.0 ± 0.0	≤1.0 ± 0.0	4.9 ± 0.3	5.0 ± 0.2	6.1 ± 0.3
CAN83	≤1.0 ± 0.0	8.3 ± 0.2	9.8 ± 0.2	4.6 ± 0.1	1.5 ± 0.0	1.5 ± 0.0
rHPIV1wt	6.1 ± 0.3	≤1.0 ± 0.0	≤1.0 ± 0.0	2.0 ± 0.2	4.4 ± 0.2 ^d	5.8 ± 0.2 ^e
rHPIV1-F ₈₃	4.7 ± 0.2	6.0 ± 0.3	8.6 ± 0.2	1.9 ± 0.2	2.9 ± 0.3 ^d	3.9 ± 0.1 ^e

a. Groups of 18 hamsters were infected IN with 10⁵ TCID₅₀ of the indicated virus or with L15 medium as a negative control.

b. Thirty-three days following the first infection, sera were collected and assayed to determine the neutralization titers against HPIV1, CAN75 and CAN83. 50 days following the first infection, six hamsters from each group were challenged IN with 10⁵ TCID₅₀ of the indicated virus and the nasal turbinates were harvested after four days.

c. Virus present in the tissue homogenates was quantified and is expressed as the mean log₁₀ TCID₅₀/g for each group ± standard error.

d. Statistically significant difference between indicated values; $p < 0.01$ (unpaired *t*-test).

e. Statistically significant difference between indicated values; $p < 0.0001$ (unpaired *t*-test)

Example 7**Construction of HMPV Recombinant Virus Expressing Protective Antigens for Both Putative Antigenic Subgroups of HMPV**

As described above, different strains of HMPV can exhibit genetic diversity, especially in the SH and G proteins, and there appear to be two major genetic subgroups. The preceding Example identified the HMPV F protein as a major neutralization and protective antigen effective against both subgroups. Thus, it is a preferred antigen for including in an HMPV vaccine. The preceding Examples showed that it is possible to achieve a high degree of protective immunity against both genetic subgroups by infection with a single HMPV from either subgroup. Furthermore, the F protein was identified as a major neutralization and protective antigen effective against both subgroups. However, it may be that optimal immunogenicity and protective efficacy will be achieved by including the SH or G protein from either or both subgroups, or by including F protein from both subgroups. In addition, the available information indicates that HMPV is a significant cause of serious respiratory tract disease early in life, approximately coincident with RSV and the PIVs. Thus, an immunogenic composition against HMPV likely would be administered at approximately the same time in life (optionally in a combinatorial formulation or coordinate immunization protocol for infants, and certain adult subjects) as immunogenic compositions directed against RSV and the PIVs. Accordingly, certain aspects of the current disclosure provide for broad coverage of an HMPV immunogenic composition, yielding increased effectiveness against multiple, antigenically-distinct strains. It is also advantageous in certain embodiments to decrease the number of separate viruses that must be administered: an immunogenic composition comprised of multiple separate viruses is more difficult to develop and formulate since each virus must be verified to be safe and immunogenic separately and in combination, and issues such as viral interference can complicate formulation. The methods of the current disclosure offer a flexibility and versatility in design of immunogenic compositions that addresses these and related problems.

In one exemplary strategy, one or more ORFs each encoding a heterologous, immunogenic protein or antigenic determinant (for example, fragment or epitope) thereof can be engineered into transcription cassettes and inserted into the genome of HMPV. For example, as illustrated in FIG. 34, an ORF encoding the G, F or SH surface protein of HMPV strain 75 is engineered to be flanked by GS and GE signals from strain 83, and is inserted into the HMPV strain 83 backbone. This results in a chimeric virus that contains an additional gene and expresses all of the genes of strain 83 as well as a surface protein antigen of strain 75. This chimeric virus therefore expresses antigens representing both HMPV antigenic subgroups. This addresses the need to broaden coverage of immunogenic compositions, as well as meeting the need to minimize the number of separate viruses that must be included in such compositions. A preferred heterologous strain HMPV gene for insertion into an HMPV backbone would be the F gene, since F has been directly identified as a major HMPV neutralization and protective antigen. A second preferred gene is the G gene, since its encoded protein appears to be highly divergent between HMPV strains, exhibiting only 70% amino

acid identity between strain 00-1 and 83 compared to 98% identity for the F protein. Similarly, the SH protein is a third preferred gene for insertion into chimeric virus, since it also is relatively divergent and exhibits 85% sequence identity between strains 00-1 and 83. The example illustrated in FIG. 34 employed the same transcription signals and genome insertion site that was used for expression of GFP. However, the methods of the current disclosure allow other signals or insertion sites to be tested. Also, the Example of FIG. 34 involved an ORF from a heterologous strain of HMPV, but the same strategy can be applied to other heterologous ORFs, such as ones from RSV, HPIV1, 2 or 3, other heterologous viruses or pathogens, and other molecules such as immunomodulatory proteins such as granulocyte-macrophage colony stimulating factor. In this way, an HMPV backbone can be used to create additional viruses bearing the surface proteins of heterologous, non-HMPVs. Chimeric viruses of the current disclosure can then be administered on their own, or can be combined with the original parent or other rHMPV to make a multi-virus or multivalent immunogenic composition.

Example 8

Methods of Screening for Compounds that Inhibit HMPV

The current disclosure demonstrates that expression of the N, P, L and M2-1 proteins alone are sufficient to direct HMPV transcription and RNA replication. This was documented in two ways. First, expression of these proteins was sufficient to direct transcription and RNA replication of a cDNA-encoded mini-replicon derived from the HMPV genome and carrying a reporter gene. Second, expression of these proteins was sufficient to launch the rescue of complete infectious HMPV from a cDNA-encoded antigenome. The antigenome is by definition the complement of the genome and is not able to serve as the direct template for transcription. Hence, both RNA replication and transcription must have occurred under the direction of the supplied proteins in order for a productive infection to be launched, indicating that N, P, L, and M2-1 are sufficient to direct all phases of HMPV RNA synthesis. Therefore, the current disclosure provides novel and readily practiced methods for screening, identifying, designing, and characterizing compounds that inhibit one or more activities of HMPV (for example, transcription, translation, growth, infectivity, virulence, immunogenicity, etc.)

In addition, the current disclosure provides new, facilitated methods of monitoring infection by complete HMPV which provide for the screening, identification and design of antiviral compounds that act at any stage in the HMPV replicative cycle. The current disclosure also provides methods for identifying the viral targets of antiviral compounds, thereby providing for improved design of compounds effective in inhibiting HMPV.

Potential antiviral compounds effective against HMPV include monoclonal or polyclonal antibodies obtained by biological or recombinant methods, peptides or protein fragments (Lambert *et al.*, Proc. Natl. Acad. Sci. USA 93:2186-2191, 1996), sugar derivatives, polyionic polymers, anti-sense RNA, double stranded RNAs capable of mediating RNA interference RNA (RNAi) (Bitko and Barik, BMC Micro. 1:34-44, 2001), and small molecule compounds (De Clercq, Antimicrob. Agents

7:193-202, 1996; Prince, Ex. Opin. Invest. Drugs 10:197-308, 2002). In general, the methods will apply to any antiviral compounds capable of inhibiting HMPV in solution, in cell extracts, in cell culture, and *in vivo*. Antiviral compounds can act, for example, by inhibiting attachment, penetration (fusion), RNA synthesis, and virion assembly, and in other cases their specific target of action may not be fully elucidated (De Clercq, Antimicrob. Agents 7:193-202, 1996; Prince, Ex. Opin. Invest. Drugs 10:197-308, 2002). Antiviral agents also can act by enhancing the host immune response, such as by stimulating the interferon-mediated antiviral state (Player *et al.*, Proc. Natl. Acad. Sci. USA 95:8874-9, 1998). In addition, an antiviral agent can be combined with an anti-inflammatory agent to effect a further reduction in disease (Prince *et al.*, J. Infect. Dis. 182:1326-1330, 2000).

10 In one embodiment of the current disclosure, rHMPV-GFP is used to screen antiviral compounds. In one exemplary strategy, rHMPV-GFP is treated with a test compound or library of compounds (for example, a compound or library of compounds prospectively including one or more antiviral agents) and compared with mock-treated control rHMPV-GFP for the ability to infect cells. Following a period of absorption, the inoculum containing the potential antiviral compound can be removed by washing if desired. Infectivity is typically assayed by observation or measurement of a detectable label or signal whose presence or level of expression or detection is correlated with one or more activities of HMPV, for example as indicated by the development of green fluorescence due to expression of the GFP marker gene by recombinant HMPV-GFP, a method that can be monitored in living cells without compromising sterility over the course of the infection. Alternatively, the antiviral agent can be included in the medium overlay for the duration of the infection. Alternatively, infection can be initiated in the absence of the compound, which can be subsequently added. In addition to identifying compounds capable of inhibiting one or more activities of HMPV, these assays can give an indication of the mode of action of a compound, such as whether it acts by directly neutralizing virus, or inhibiting subsequent gene expression, or inhibiting viral spread. While GFP is provided as an exemplary label or marker, a wide range of useful "reporter sequences" (for example, reporter genes) and other sequences that direct expression of detectable labels and markers are known in the art and can be readily employed as detectable "signal" agents whose presence, level of activity or expression correlate with one or more selected activities of HMPV. Exemplary alternative markers and/or labels in this context include, *inter alia*, chloramphenicol acetyl transferase, luciferase, secreted alkaline phosphatase, and a large number of other known reporter sequences routinely used in the art. This provides the ability to design a reporter virus or subviral construct that will be useful in a variety of high throughput screening protocols.

35 Within related aspects of the current disclosure, the availability of rHMPV variants that each lack one or more of the viral ORFs, as described above, provides particularly useful tools and methods to identify possible viral targets of antiviral compounds. For example, if a compound inhibits the infectivity of complete HMPV, it will be tested (for example, for its efficacy and/or target gene or protein specificity) against a panel of rHMPV lacking various genes, such as the Δ SH, Δ G, Δ SH/G, and Δ M2-2 variants mentioned previously. An alternative and complementary method of

identifying targets for an antiviral compound involves forcing the virus to grow in the presence of the compound (present at minimal or suboptimal inhibitory concentrations if necessary). Under these conditions, it is expected that a large panel of useful variants will emerge that have developed increased resistance to the subject inhibitory compound. Sequence analysis of such variants can
5 identify mutations that are potentially responsible for the increased resistance. By the methods of this current disclosure, mutations suspected of conferring increased resistance can be introduced individually or in combinations into rHMPV, and the resistant rHMPV expressing a convenient reporter gene such as GFP will provide yet additional tools and methods to facilitate other phases of HMPV control and antiviral development. For example, the introduction of mutations identified in
10 resistant viruses provides a method of directly confirming that they confer resistance and thus distinguishes them from adventitious mutation that occur during passage. This provides direct identification of viral genes involved in drug resistance, and is an important tool for further drug development. As another example, as noted above, HMPV-GFP can be used to assay quickly and accurately to detect antibodies to HMPV. This can be advantageous in studies to develop monoclonal
15 or polyclonal antibodies capable of neutralizing HMPV as immunoprophylactic or therapeutic agents. This also has applications in epidemiologic studies for example to determine the serostatus of individuals in a population. Furthermore, it provides a facilitated method to monitor changes in the level of HMPV antibodies in experimental animals or clinical subjects, such as in response to experimental immunogenic compositions, or in situations where the efficacy of an antiviral compound is being evaluated. Also, the expression of a reporter gene such as GFP provides a useful marker to
20 detect and quantify HMPV infection and immunity in individuals, including experimental animals, thereby providing an assay for viral disease detection, tropism and/or to identify and evaluate additional antiviral agents.

In other embodiments of the current disclosure, rHMPV expressing a convenient reporter
25 gene can be readily employed within screening compositions and methods based on cell-free *in vitro* assays. For example, a nucleocapsid preparation can be made from cells infected with rHMPV expressing a reporter gene, such as alkaline phosphatase or some other sensitively-detected enzyme or marker. Replicate *in vitro* assays can be constructed and used to screen compounds for antiviral activity in a high throughput format designed to detect the expressed reporter gene. This can be
30 based on the expression of a convenient reporter gene or, alternatively, can be based on the detection of RNA representing the marker gene or one or more HMPV genes. For example, mRNA produced in such an *in vitro* reaction mixture can be captured *in situ* by matrix-bound oligodT and, following washing to remove the reaction mixture, the resulting hybrids can be detected by secondary hybridization with probes tagged with reporter enzymes or ligands. Such assays can be further
35 modified to specifically target, for example, products of RNA replication or read-through of gene junctions as a measure of altered transcription.

The identification of the N, P, L, and optionally, M2-1 as proteins useful and/or sufficient to direct HMPV transcription and RNA replication provides additional new methods for screening

compounds for anti-viral activity. In one embodiment, these four proteins can be expressed intracellularly together with a mini-replicon that encodes a reporter gene, resulting in reconstituted HMPV transcription and RNA. This can be used to screen for compounds, particularly those that are effective against HMPV transcription, RNA replication, and nucleocapsid assembly and function.

- 5 Alternatively, the N, P, L, and M2-1 proteins can be expressed at high levels, using recombinant baculovirus, vaccinia virus, bacteria or any other suitable expression system, and purified to use in reconstituted assays such as *in vitro* RNA synthesis, protein-protein interactions, or protein-RNA interactions. Alternatively, the N, P, L and M2-1 proteins can be tagged recombinantly by the incorporation of, for example, a hexa-Histidine tag or an epitope recognized by a monoclonal
- 10 antibody, such as the well known FLAG tag (Nilsson *et al.*, Prot. Exp. Pur. 11:1-16, 1997). These provide the basis for purification of these components by affinity chromatography, facilitating the development of *in vitro* assays. In addition, when such tagged genes are expressed from the context of infection by recombinant HMPV, it provides a method of isolating and identifying possible cellular and viral binding partners. Such binding partners can be identified by a variety of methods.
- 15 For example, in some cases, binding partners will be present in sufficient quantity that they can be visualized by gel electrophoresis and identified by conventional methods of protein sequencing. Low abundance binding partners can be identified from complex mixtures by mass spectrometric methods coupled with trypsin cleavage and protein sequencing. The identification of viral and cellular binding partners provides additional information to guide the development of compounds that inhibit HMPV.

- 20 While this disclosure has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and it is intended that the disclosure may be practiced otherwise than as specifically described herein. Accordingly, this disclosure includes all modifications encompassed within the spirit and scope of the disclosure as defined by the claims below.

CLAIMS

We claim:

- 5 1. An isolated recombinant human metapneumovirus (rHMPV), comprising
a partial or complete, recombinant HMPV genome or antigenome comprising one or
more attenuating nucleotide modifications, and
a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large
polymerase protein (L).
- 10 2. The rHMPV of claim 1, wherein the rHMPV is replication competent.
3. The rHMPV of claim 1, wherein the recombinant HMPV genome or antigenome
further comprises a detectable heterologous sequence encoding a polypeptide.
4. The rHMPV of claim 3, wherein the detectable heterologous sequence encodes a
reporter.
- 15 5. The rHMPV of claim 4, wherein the reporter comprises green fluorescent protein
(GFP).
6. The rHMPV of claim 3, wherein the detectable heterologous sequence is operably
linked to HMPV transcription gene start and gene end signals.
7. The rHMPV of claim 1, wherein the one or more attenuating nucleotide
20 modifications comprises a partial or complete deletion of one or more rHMPV SH, G, M2-1,
M2-2, or M2 ORFs or one or more nucleotide substitutions that reduces or ablates expression of
the one or more rHMPV SH, G, M2-1, M2-2, or M2 ORFs.
8. The rHMPV of claim 7, wherein the one or more attenuating nucleotide
modifications comprises a partial or complete deletion of one or more of the rHMPV SH, G,
25 M2-1, M2-2, or M2 ORFs, such that a functional protein is not produced.
9. The rHMPV of claim 7, wherein the one or more attenuating nucleotide
modifications comprises a partial or complete deletion of a rHMPV SH ORF, such that a wild
type SH protein is not produced.

10. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a SH ORF of SEQ ID NO: 1, such that a SH protein comprising a sequence set forth as SEQ ID NO: 5 is not produced.

5 11. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV G ORF, such that a wild type G protein is not produced.

12. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a G ORF of SEQ ID NO: 1, such that a G protein comprising a sequence set forth as SEQ ID NO: 6 is not produced.

10 13. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV SH and G ORFs, such that a wild type SH protein and a wild type G protein are not produced.

14. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a SH ORF and a G ORF of SEQ ID
15 NO: 1.

15. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises one or more nucleotide substitutions that reduces or ablates expression of a rHMPV M2-2 ORF.

20 16. The rHMPV of claim 15, wherein the one or more nucleotide substitutions that reduces or ablates expression of the rHMPV M2-2 ORF comprises one or more nucleotide substitutions that ablates one or more potential translation initiation codons of the rHMPV M2-2 ORF or introduces one or more in-frame stop codons into the r HMPV M2-2 ORF.

17. The rHMPV of claim 16, wherein the one or more nucleotide substitutions comprises substitutions of one or more nucleic acids of a rHMPV sequence set forth as SEQ ID
25 NO: 1.

18. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV M2-2 ORF, such that a wild type M2-2 protein is not produced.

19. The rHMPV of claim 7, wherein the one or more attenuating nucleotide
30 modifications comprises a partial or complete deletion of a M2-2 ORF of SEQ ID NO: 1.

20. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises one or more nucleotide substitutions that reduces or ablates expression of a rHMPV M2-1 ORF, such that a wild type M2-1 protein is not produced.

5 21. The rHMPV of claim 20, wherein the one or more nucleotide substitutions that reduces or ablates expression of the rHMPV M2-1 ORF comprises one or more nucleotide substitutions that ablates the translation initiation codon of the rHMPV M2-1 ORF and further ablates additional ATG triplets in each reading frame of the rHMPV M2-1 ORF.

22. The rHMPV of claim 21, wherein the one or more nucleotide substitutions comprises substitutions at one or more positions of SEQ ID NO: 1.

10 23. The rHMPV of claim 7, wherein the one or more attenuating nucleotide modifications comprises a partial or complete deletion of a rHMPV M2 ORF.

24. The rHMPV of claim 23, wherein the partial or complete deletion comprises a partial or complete deletion of the M2 ORF of SEQ ID NO: 1.

15 25. The rHMPV of claim 1, wherein the one or more attenuating nucleotide modifications produces at least one desired phenotypic change in the rHMPV, wherein the phenotypic change comprises at least one of a change in growth properties in cell culture, a change in growth properties or virulence in the upper or lower respiratory tract of a mammalian host, a change in viral plaque size, a change in sensitivity or adaptation to temperature, a change in cytopathic effect, a change in the efficiency of transcription or genome replication, a change
20 in sensitivity to interferon, a change in the efficiency of expression of one or more genes, or a change in immunogenicity.

26. The rHMPV of claim 25, wherein the one or more attenuating nucleotide modifications produces a change in viral growth in the upper respiratory tract, lower respiratory tract, or both, such that viral growth is attenuated by about 50-100 fold or greater, compared to
25 growth of the corresponding wild type HMPV strain.

27. The rHMPV of claim 1, wherein the one or more attenuating nucleotide modifications comprises one or more nucleotide substitutions that produce one or more amino acid substitutions in a M2-1 or a L protein in the rHMPV.

28. An isolated, replication competent recombinant human metapneumovirus
30 (rHMPV), comprising a partial or complete, recombinant HMPV genome or antigenome, a

major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L), wherein the genome or antigenome of the rHMPV is rearranged such that an order of one or more genes or genome segments in the recombinant HMPV genome or antigenome is altered as compared to a wild type HMPV.

5 29. The rHMPV of claim 28, wherein the order of a SH, G, or F gene or genome segment is altered in the rHMPV genome or antigenome.

 30. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a SH gene or genome segment and a G gene or genome segment inserted after a M gene and before a F gene of the rHMPV genome or antigenome.

10 31. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises at least two copies of a SH gene or genome segment and at least two copies of a G gene or genome segment inserted after a M gene and before a F gene of the rHMPV genome or antigenome.

 32. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a F gene or genome segment inserted after a 3' leader sequence and before a N gene of the
15 rHMPV genome or antigenome.

 33. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a G gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

 34. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a
20 F gene or genome segment and a G gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

 35. The rHMPV of claim 28, wherein the rHMPV genome or antigenome comprises a G gene or genome segment and a F gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

25 36. The rHMPV of claim 1, wherein the one or more attenuating nucleotide modifications comprises inserting one or more additional copies of one or more rHMPV G or F genes or genome segments in the rHMPV genome or antigenome.

 37. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises one or more additional copies of a rHMPV G gene or genome segment, a F gene or genome

segment, or both, inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

38. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises a single additional copy of a rHMPV G gene or genome segment inserted after a 3' leader
5 sequence and before a N gene of the rHMPV genome or antigenome.

39. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises a single additional copy of a rHMPV F gene or genome segment inserted after a 3' leader sequence and before a N gene of the rHMPV genome or antigenome.

40. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises
10 one additional copy of the rHMPV G gene and one additional copy of the rHMPV F gene in the order G-F.

41. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises one additional copy of the recombinant HMPV G gene and one additional copy of the recombinant HMPV F gene in the order F-G.

42. The rHMPV of claim 36, wherein the rHMPV genome or antigenome comprises
15 one additional copy of a rHMPV G gene or genome segment and two additional copies of the rHMPV F gene or genome segment in the order G-F-F.

43. The rHMPV of claim 1, wherein the rHMPV genome or antigenome further
20 comprises one or more heterologous genes or genome segments from a different paramyxovirus to form a chimeric recombinant HMPV genome or antigenome.

44. The rHMPV of claim 43, wherein the rHMPV genome or antigenome comprises one or more N, P, or M genes from a different paramyxovirus.

45. The rHMPV of claim 44, wherein the paramyxovirus comprises avian metapneumovirus.

50. The rHMPV of claim 1, wherein the rHMPV genome or antigenome further
25 comprises one or more rHMPV genes or genome segments from a different subgroup of HMPV to form a chimeric recombinant HMPV genome or antigenome.

51. An immunogenic composition comprising an immunogenically effective amount of any one of the isolated, replication competent recombinant human metapneumoviruses of claims 1-50 in a pharmaceutically acceptable carrier.

5 52. A method for inducing an immune response in a subject against human metapneumovirus, comprising administering to the subject a therapeutically effective amount of any one of the isolated, replication competent recombinant human metapneumoviruses of claims 1-50, thereby inducing an immune response in the subject against human metapneumovirus.

53. The method of claim 52, wherein the recombinant human metapneumovirus is administered in a dose of 10^3 to 10^7 PFU.

10 54. The method of claim 53, wherein the recombinant human metapneumovirus is administered to the upper respiratory tract.

55. The method of claim 52, wherein the recombinant human metapneumovirus is administered by spray, droplet or aerosol.

15 56. An isolated, replication competent recombinant virus comprising a paramyxovirus genome or antigenome and a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L), and one or more recombinant genes or genome segments from human metapneumovirus.

57. The recombinant virus of claim 56, comprising a human metapneumovirus F gene.

20 58. The recombinant virus of claim 56, wherein the virus is an influenza virus or a parainfluenza virus.

59. An expression vector comprising an operably linked transcriptional promoter, a partial or complete, recombinant human metapneumovirus (rHMPV) genome or antigenome, and a transcriptional terminator.

25 60. The expression vector of claim 59, wherein the rHMPV genome or antigenome comprises one or more attenuating nucleotide modifications.

61. A method of screening an antiviral compound for inhibition of a biological activity of a human metapneumovirus, comprising

providing a recombinant human metapneumovirus (rHMPV) comprising a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L),

and a partial or complete, recombinant HMPV genome or antigenome modified to incorporate a detectable heterologous sequence encoding a polypeptide correlated with the biological activity upon expression of the heterologous sequence;

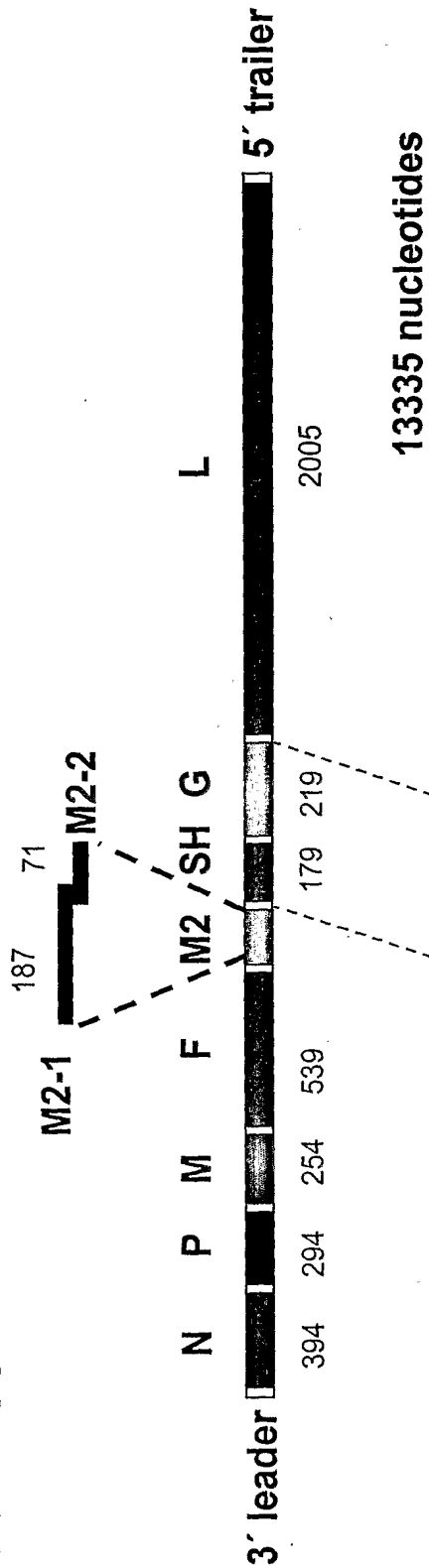
5 exposing a test sample comprising the rHMPV or a host cell amenable to infection by HMPV to a test compound or library of test compounds that prospectively includes one or more antiviral agents capable of inhibiting the biological activity of HMPV;

providing a control sample comprising the rHMPV or host cell under suitable control conditions in the absence of the test compound or library of test compounds; and

10 detecting heterologous sequence in the test and control samples to determine an increase or decrease of the biological activity in the test sample compared to the control sample to determine the presence or absence of the antiviral compound in the test sample.

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HMPV 83



RSV A2

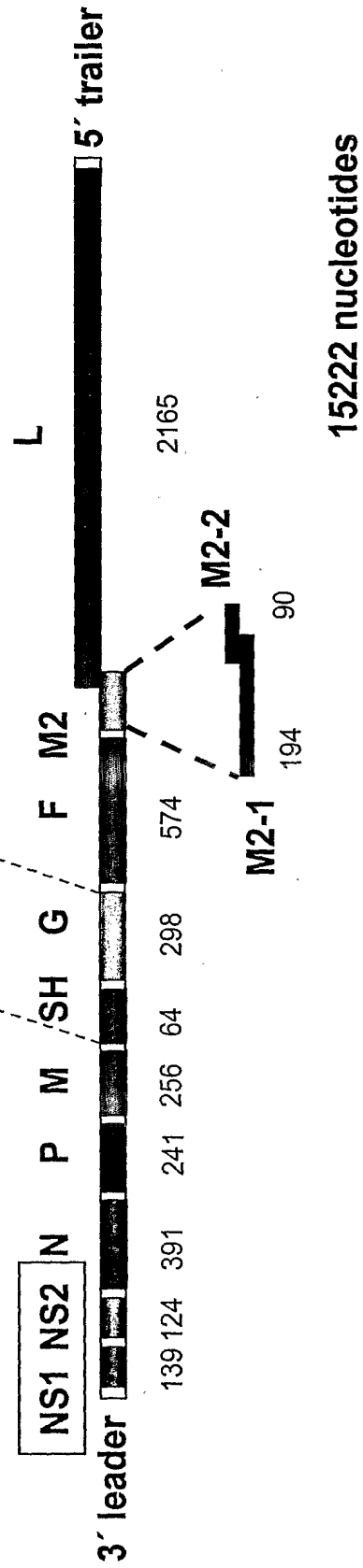


Fig. 1

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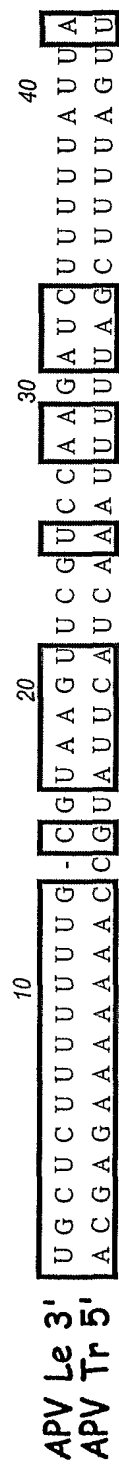
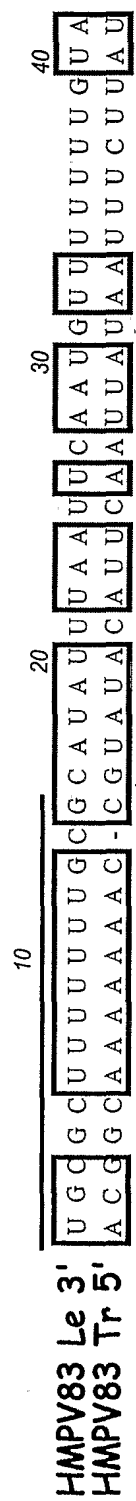


Fig. 3

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Examples of differences between HMPV 83 and HMPV 00-1

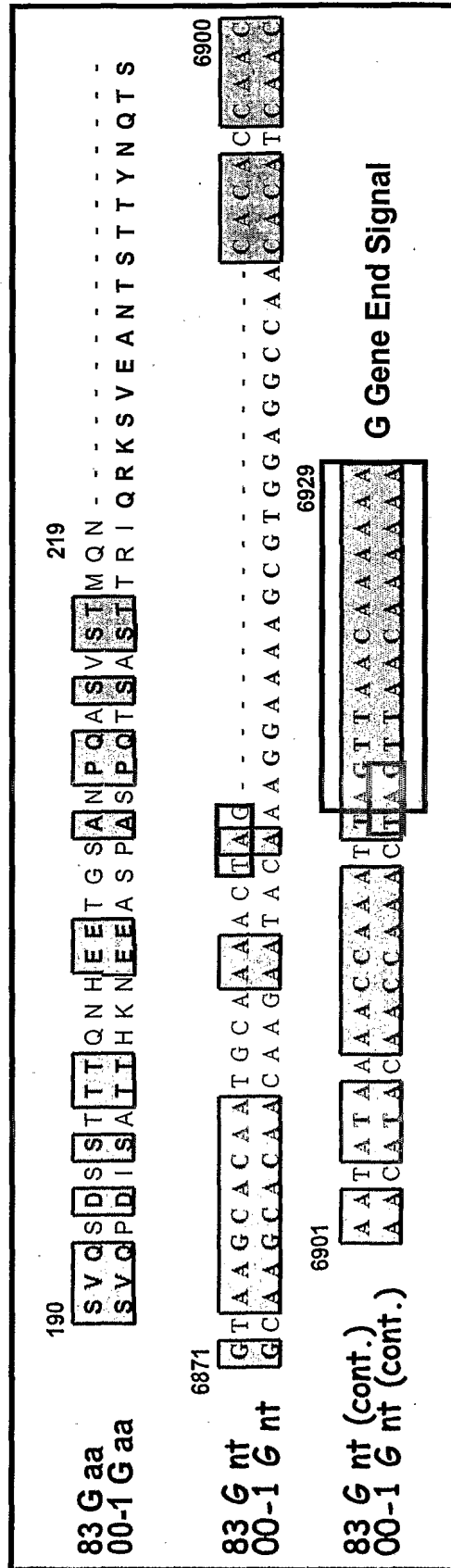


Fig. 4

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Amino acid identity between HMPV83 and other Pneumoviruses for the indicated proteins

	N	P	M	F	M 2-1	M 2-2	G	SH	L
HMPV 00-1	99	95	99	98	98	96	70	85	99
HMPV 97-82	95	85	97	94	ND	ND	ND	ND	ND
APV A	70	58	77	68	73	25	12	20	64
APV B	69	53	76	67	71	27	ND	20	ND
APV C	88	68	87	81	83	56	ND	ND	ND
HRSV A2	41	31	38	36	36	12	15	6	46
HRSV B	41	31	37	35	35	8	15	6	46
BRSV	41	31	37	37	35	14	19	10	46
PVM	45	28	38	40	36	12	ND	8	ND

ND: Comparison not done, usually because sequence was unavailable

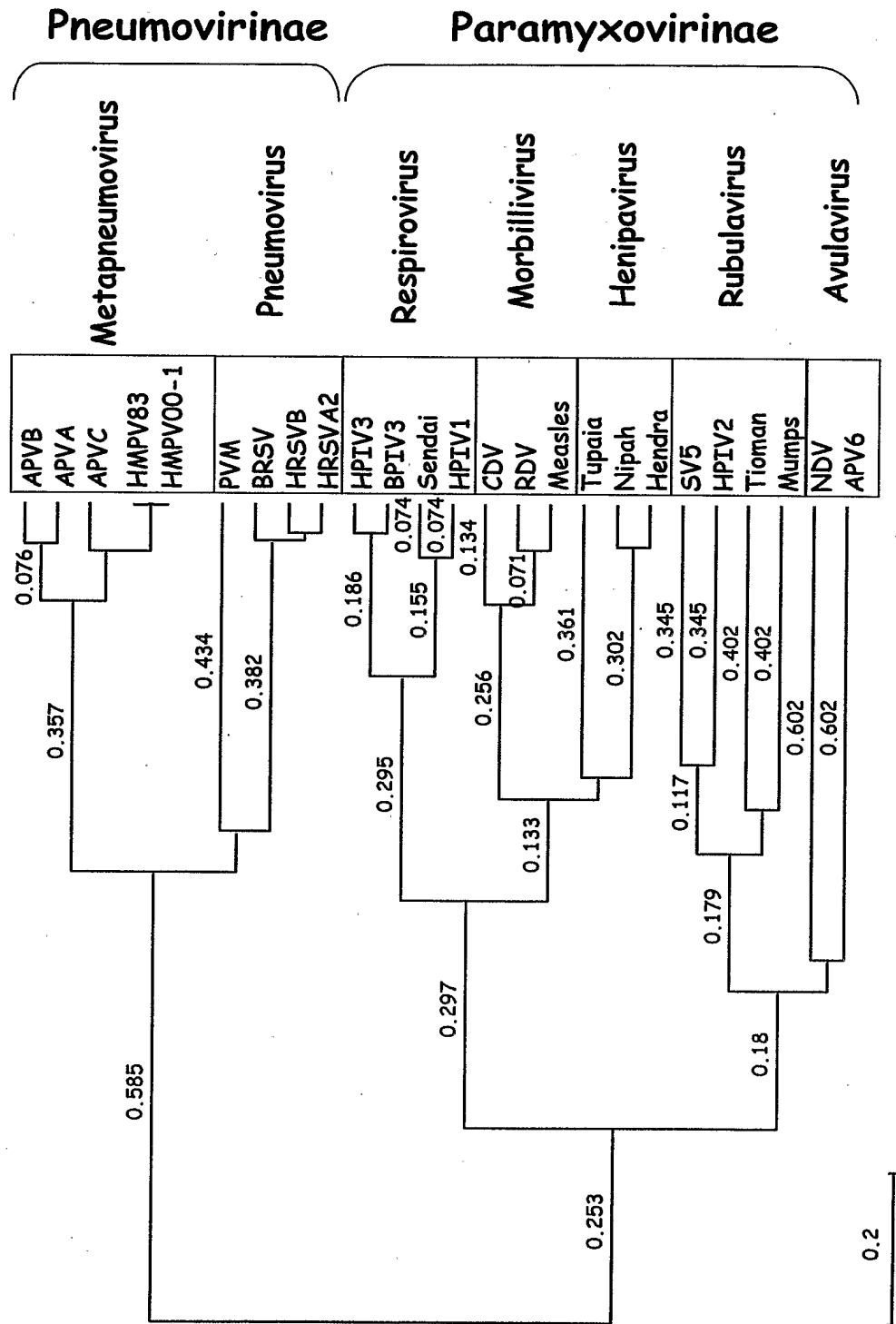


Fig. 6

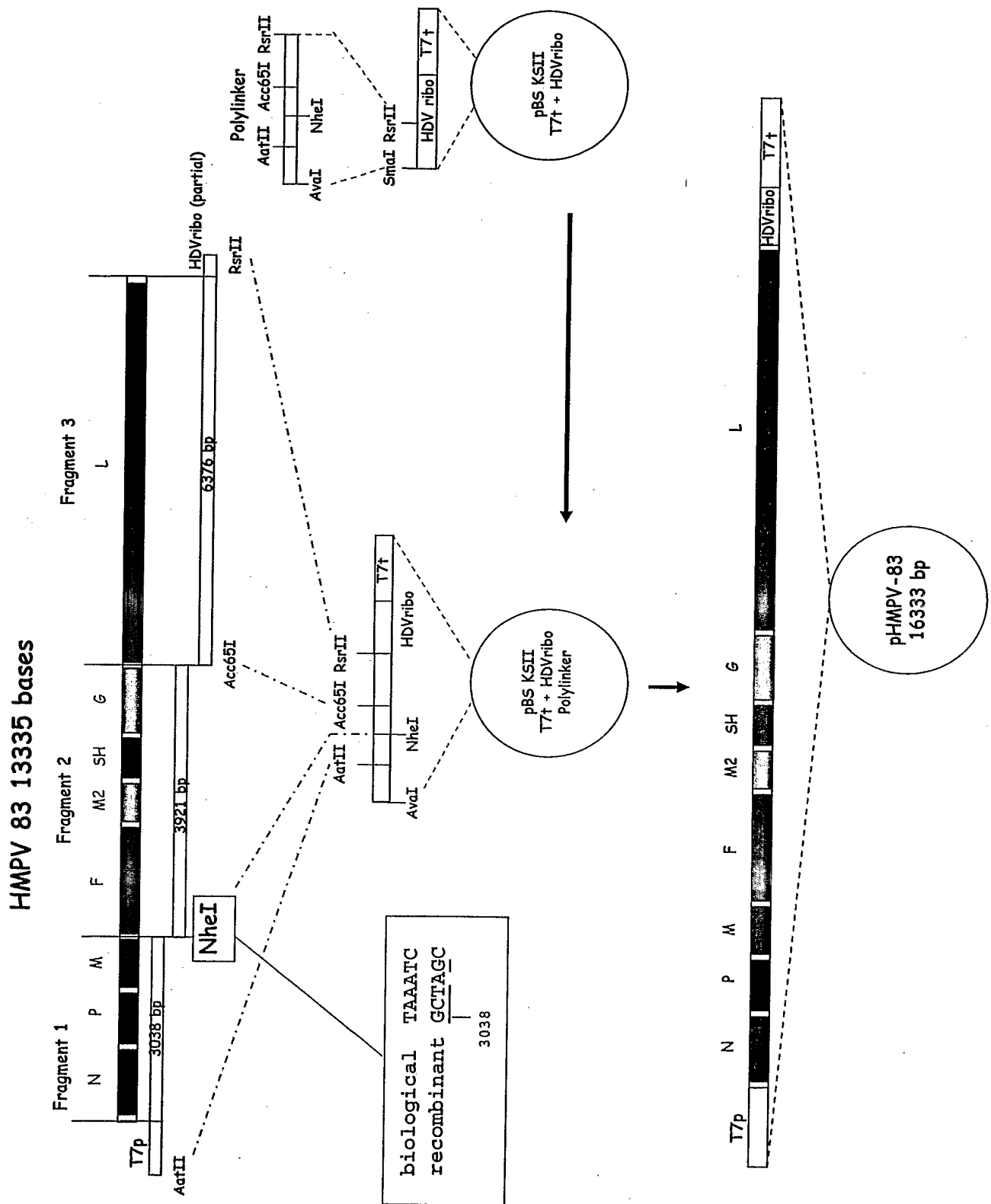


Fig. 7

Gene Start Signal

	1	5	10	15
N	tacaaaaaacat	GGGACAAAGTGAAA	ATGtctcttc	
P	taattaaaaagt	GGGACAAAGTGAAA	ATGtcattcc	
M	aaaaataaaaaat	GGGACAAAGTGAAA	ATGgagtcct	
F	atcaatcaagaac	GGGACAAATATAAA	ATGtcttgga	
M2	taaaataaaaattt	GGGACAAATCATA	ATGtctcgca	
SH	aacacatcagagt	GGGATAAGTGACA	ATGataacat	
G	aaaacaaaaatat	GGGACAAAGTAGTT	ATGgaggtga	
L	aaacagcatccaa	GAGACAAATAGCA	ATGgatcctc	

CONSENSUS

...at **GGGACAAAGTGAAA** **ATG**tc...

gc	A	T	A	AGTT	ga
ta			C	C	at

Gene End Signal

	1	5	10
N	ttatg	AGTAATTAAAAA	gt
P	tatgt	AGTTTAATAAAAA	taaaaaat
M	atatt	AGTTATATAAAAA	tcaagttagaat a
F	cagtt	AGTTAATTAAAAA	taaaataaaatt t
M2	actta	AGTTAGTAAAAA	cacatcagagt
SH	agttt	AGTTATTTTAAAA	tatttgagaata g
G	aaatt	AGTTAACAAAAAA	tacgagatagct c
L	atgat	AGTTAATTAAAA	ttaaaaaattaaaa a

CONSENSUS

...tt **AGTTAATTAAAA** ta...

ag	ATTAAT	. cc
ga	GC	gt

Fig. 8

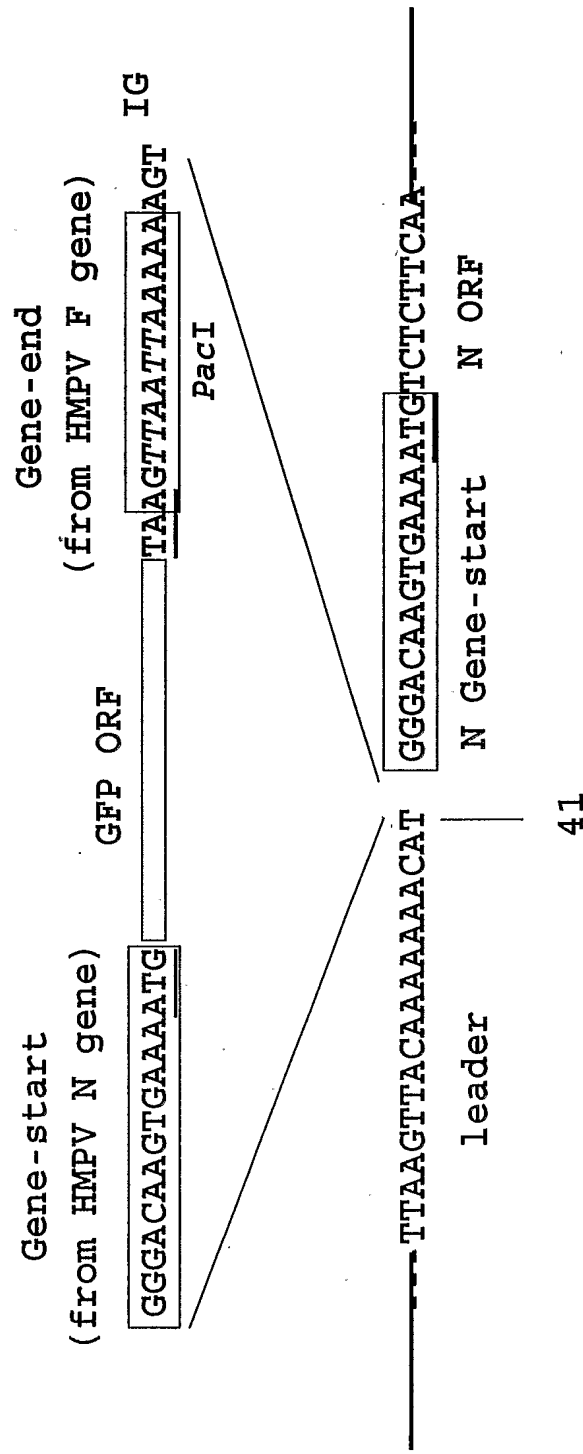


Fig. 9

10/66

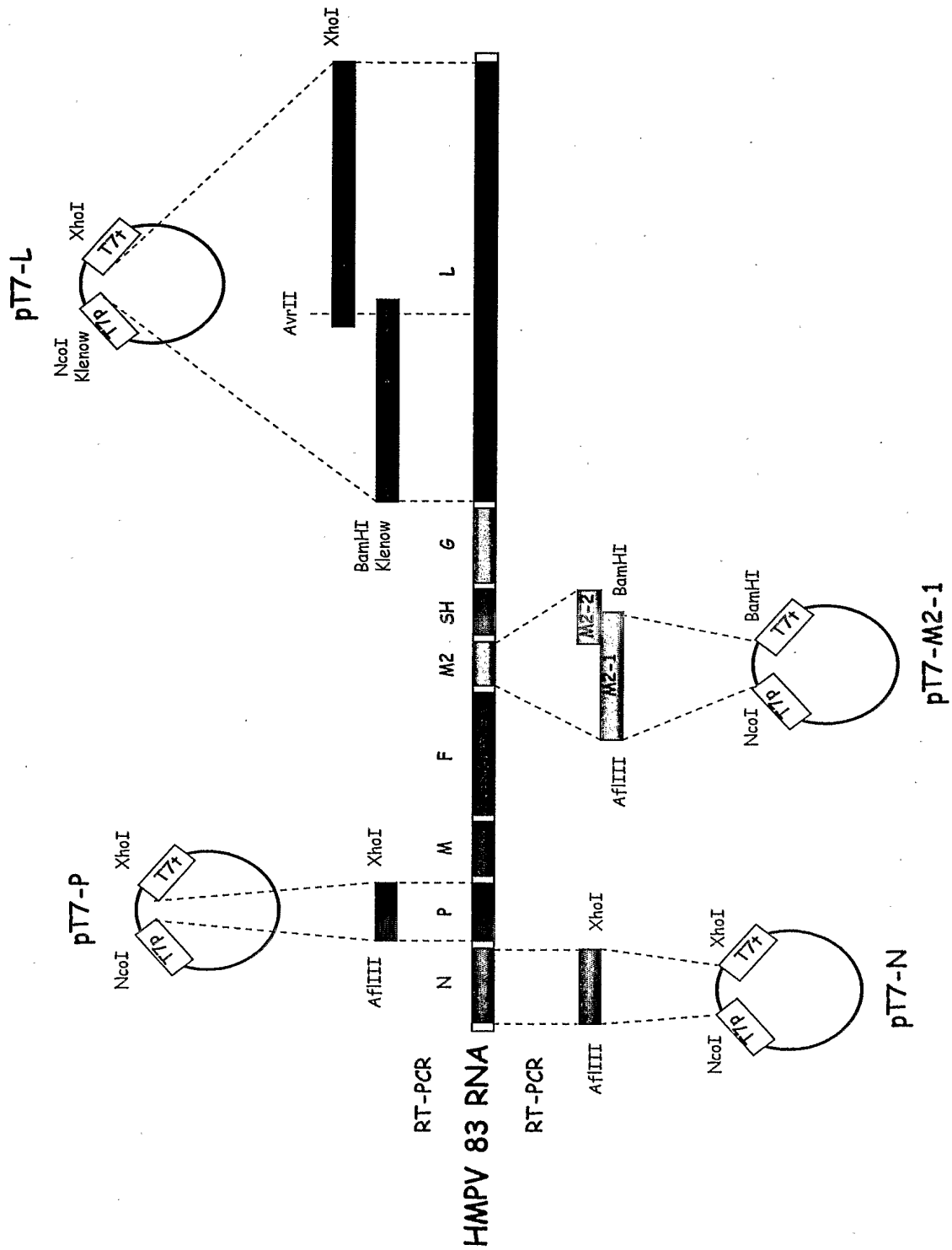


Fig. 10

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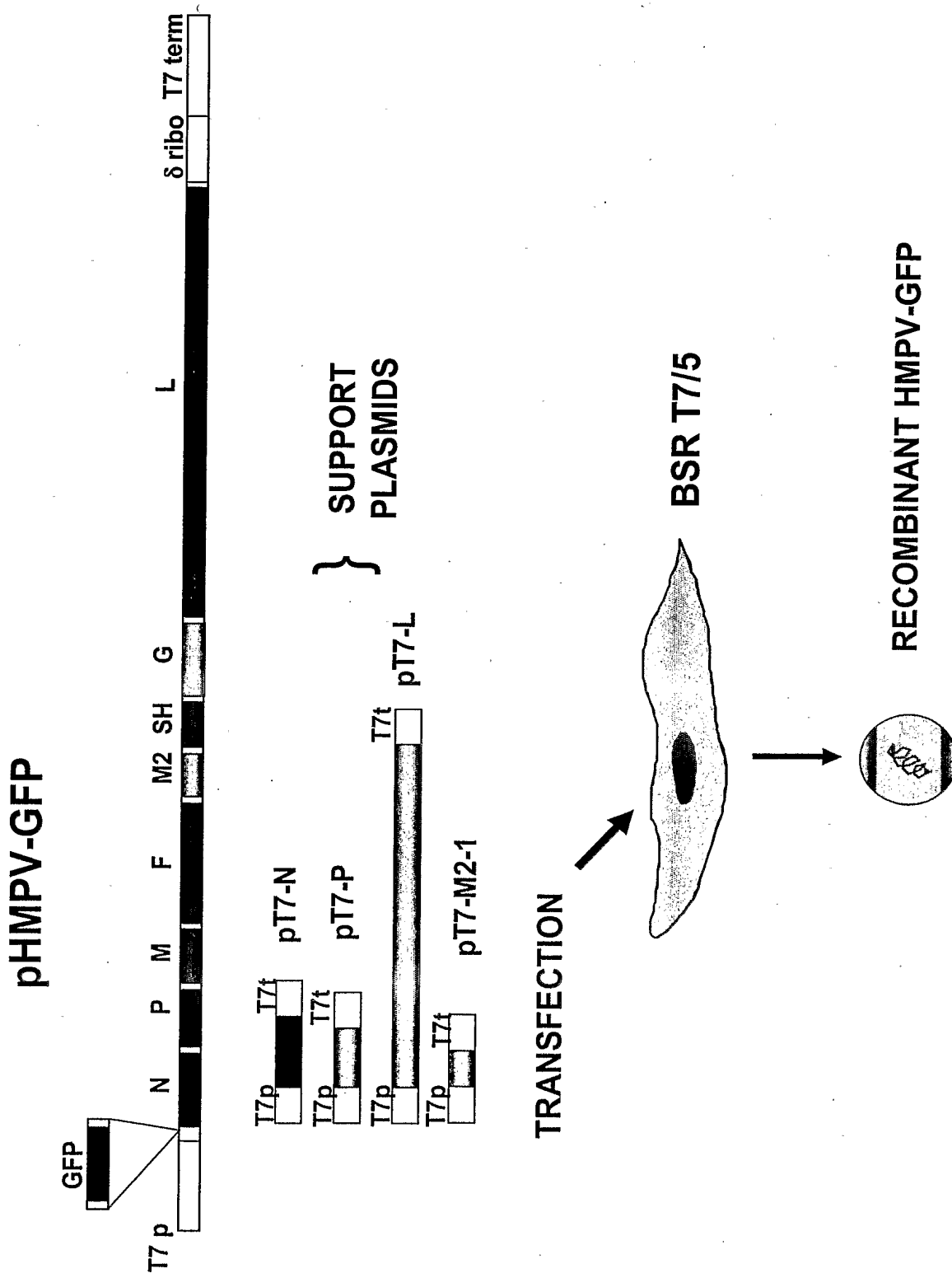


Fig. 11

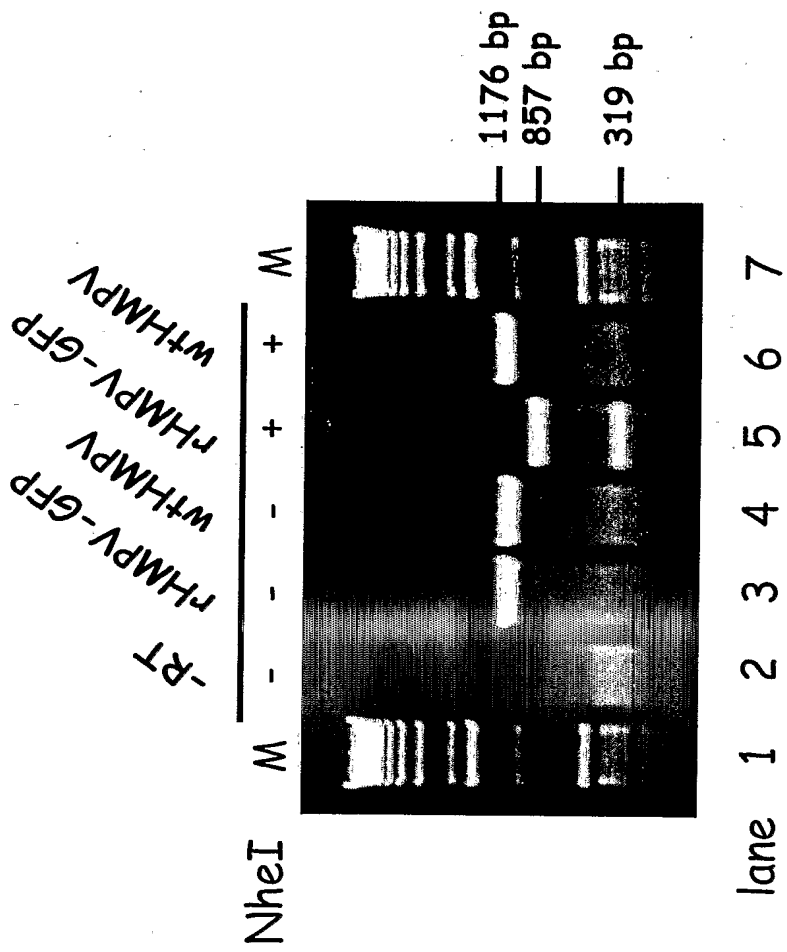


Fig. 12A

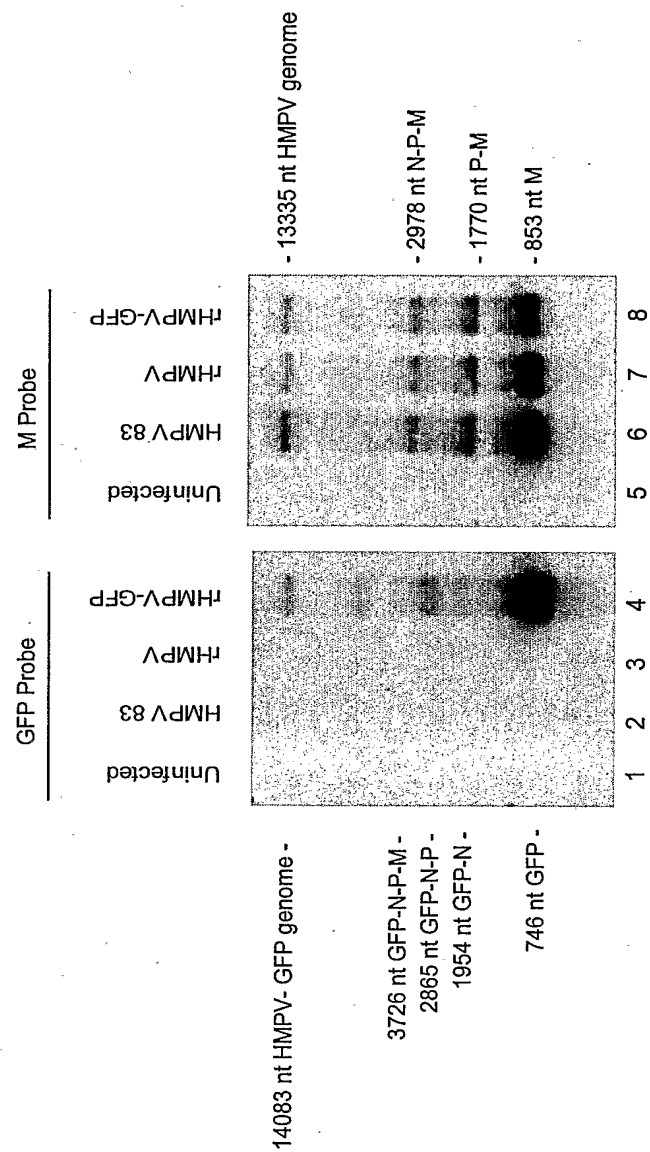


Fig. 12B

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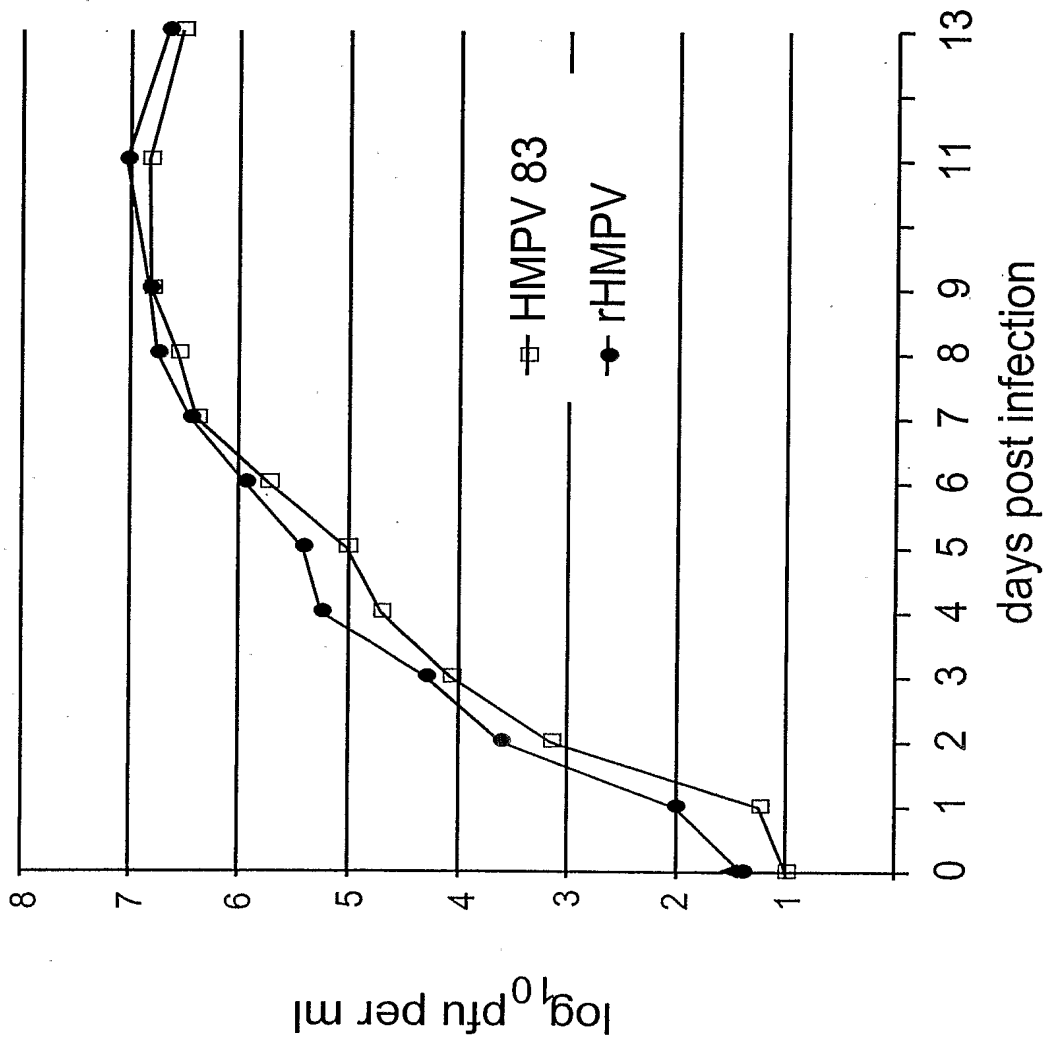


Fig. 13

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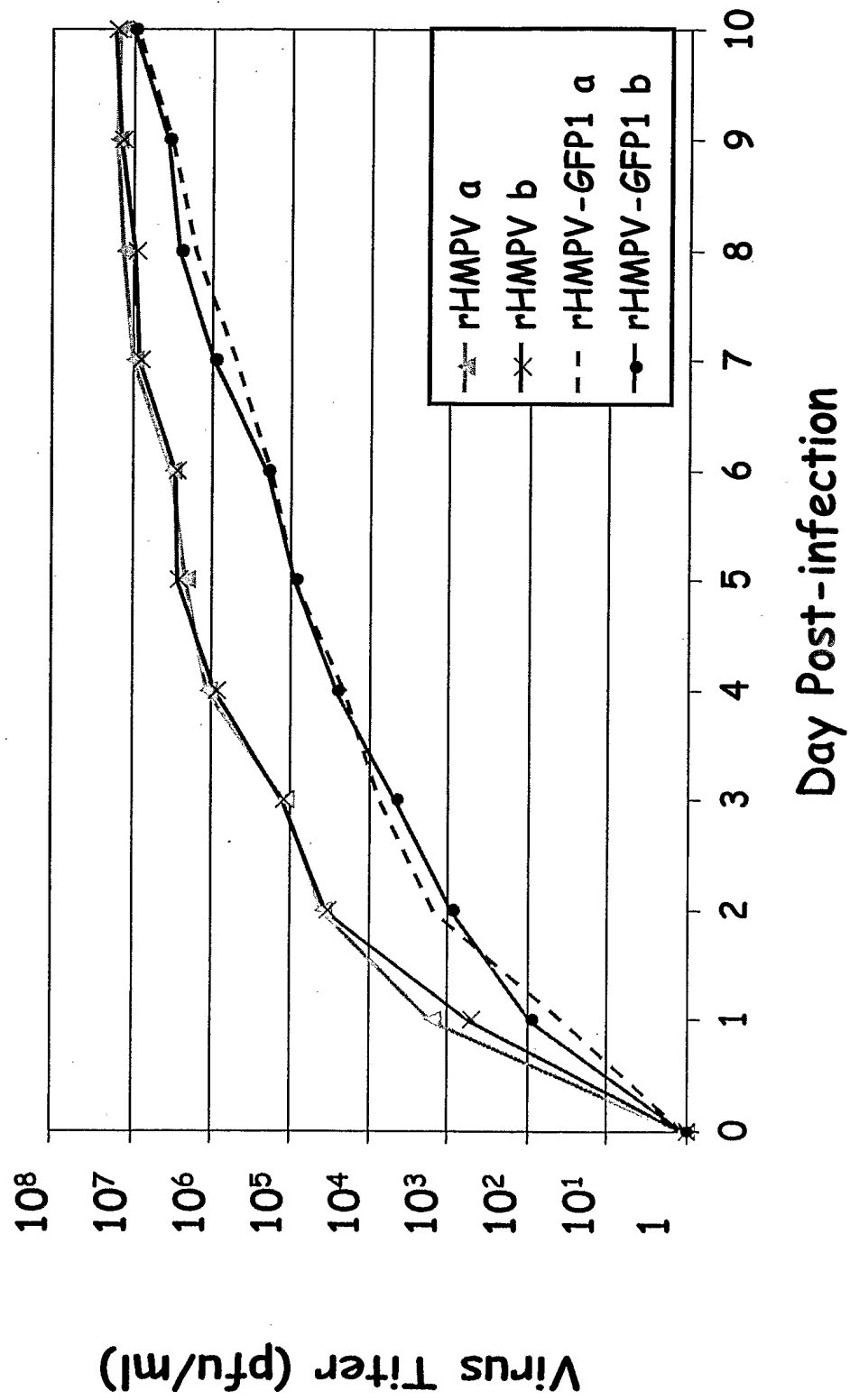


Fig. 14

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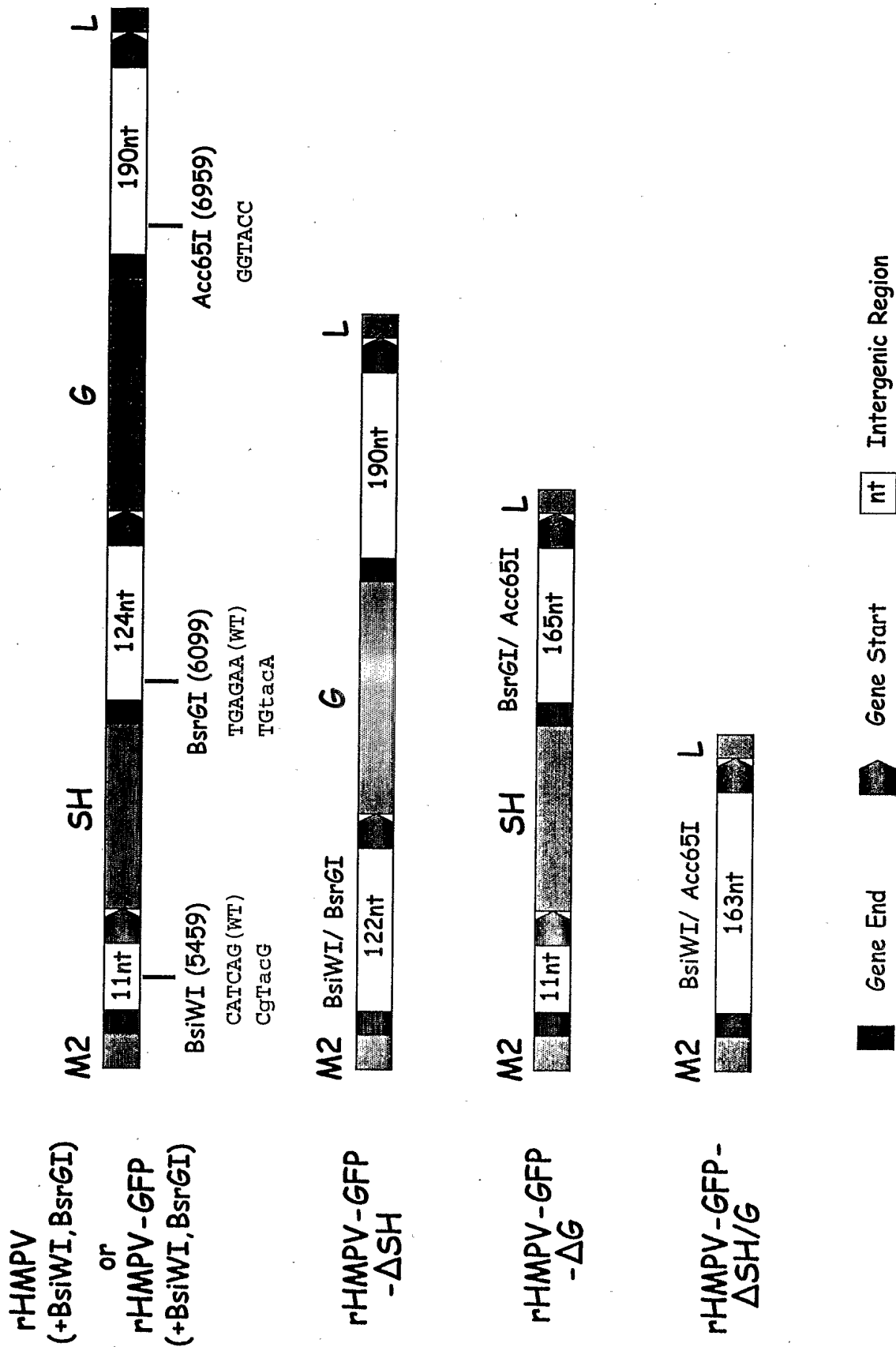


Fig. 15

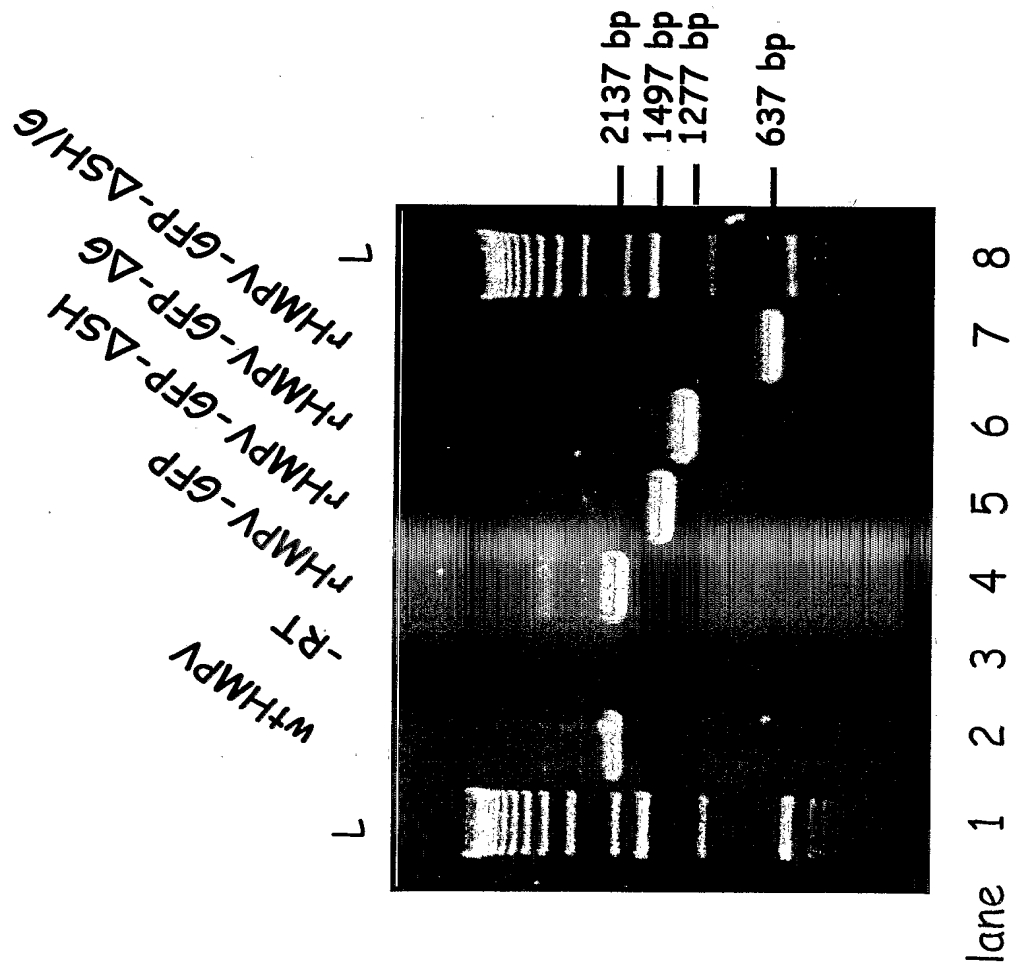


Fig. 16

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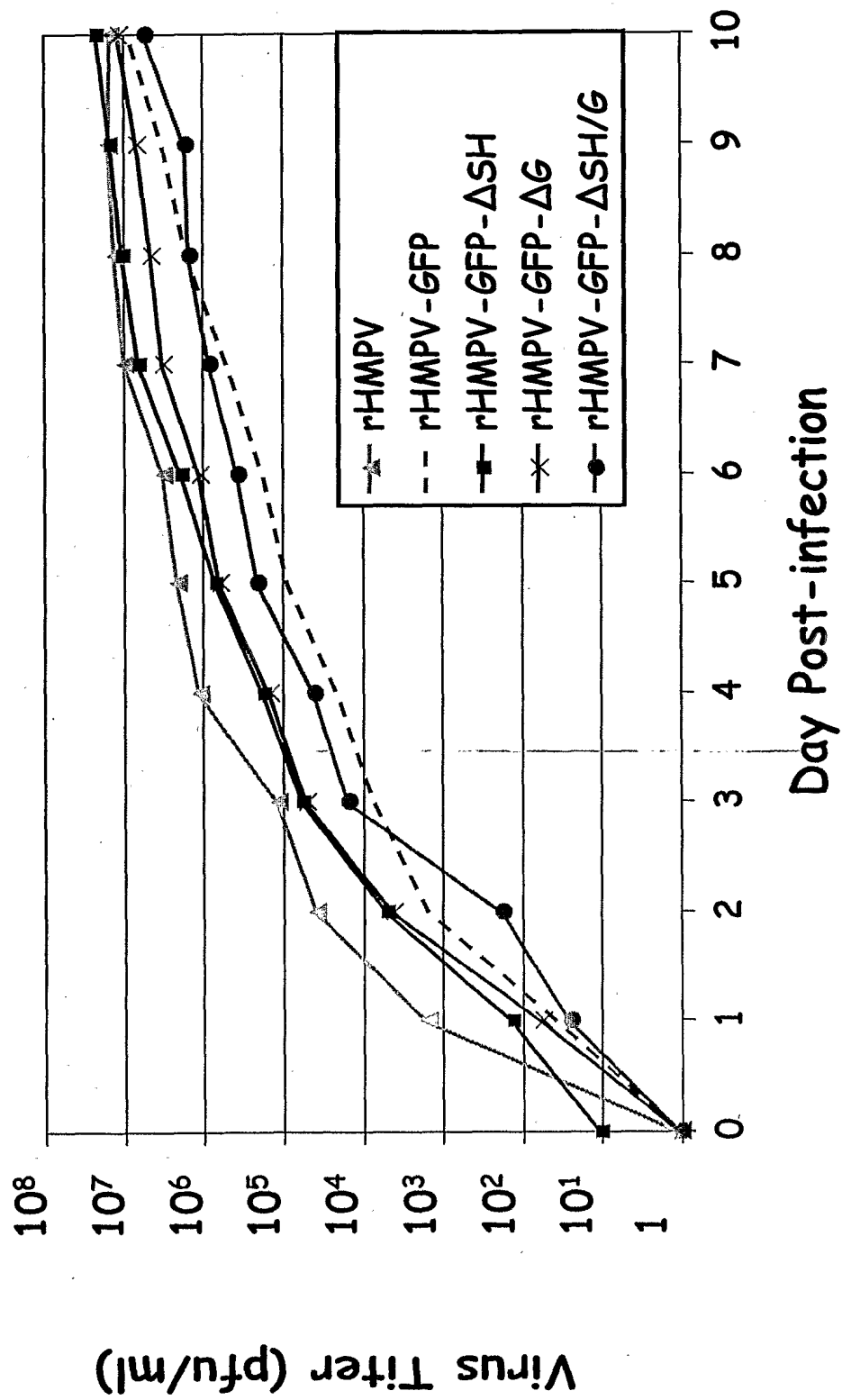


Fig. 17A

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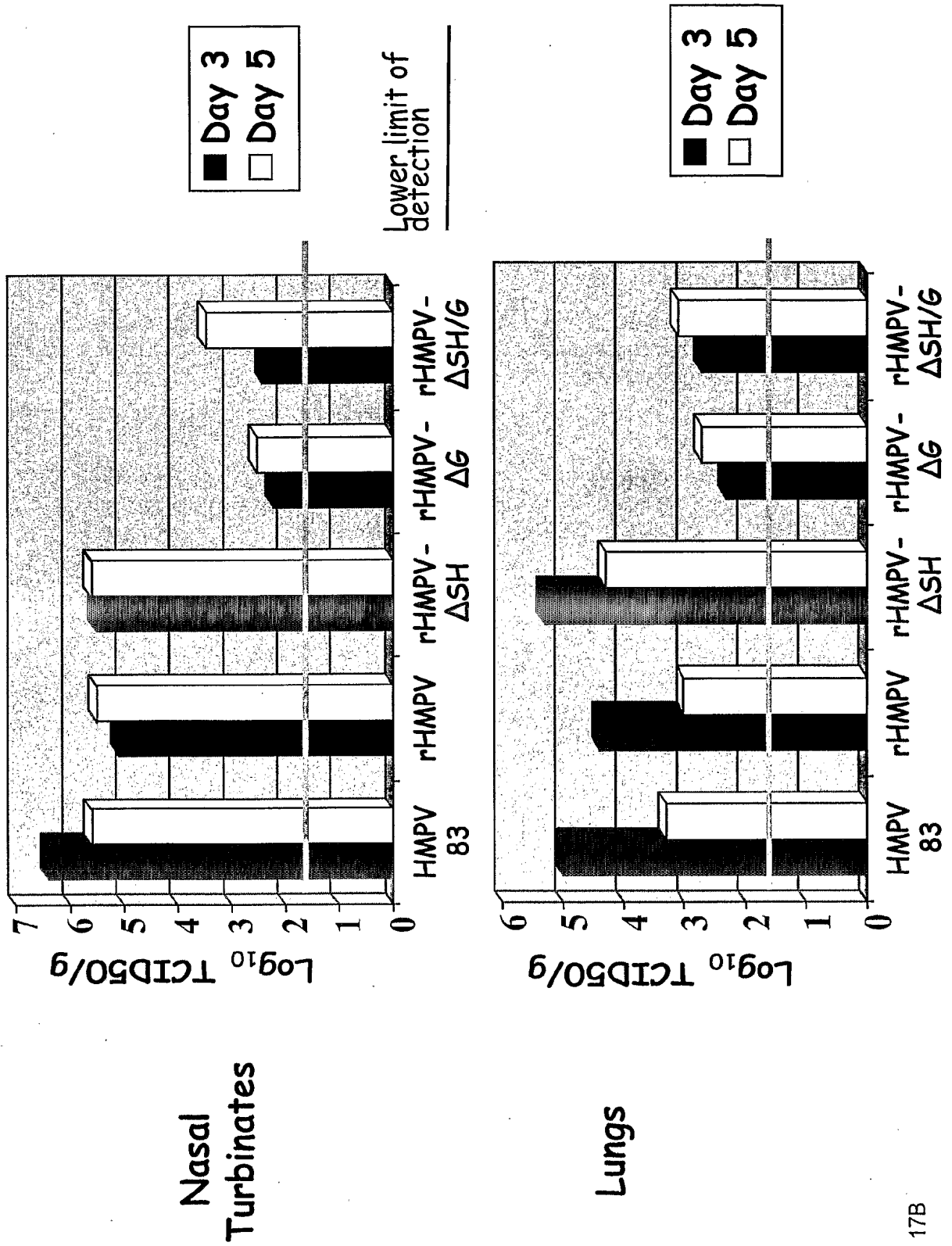


Fig. 17B

20/66

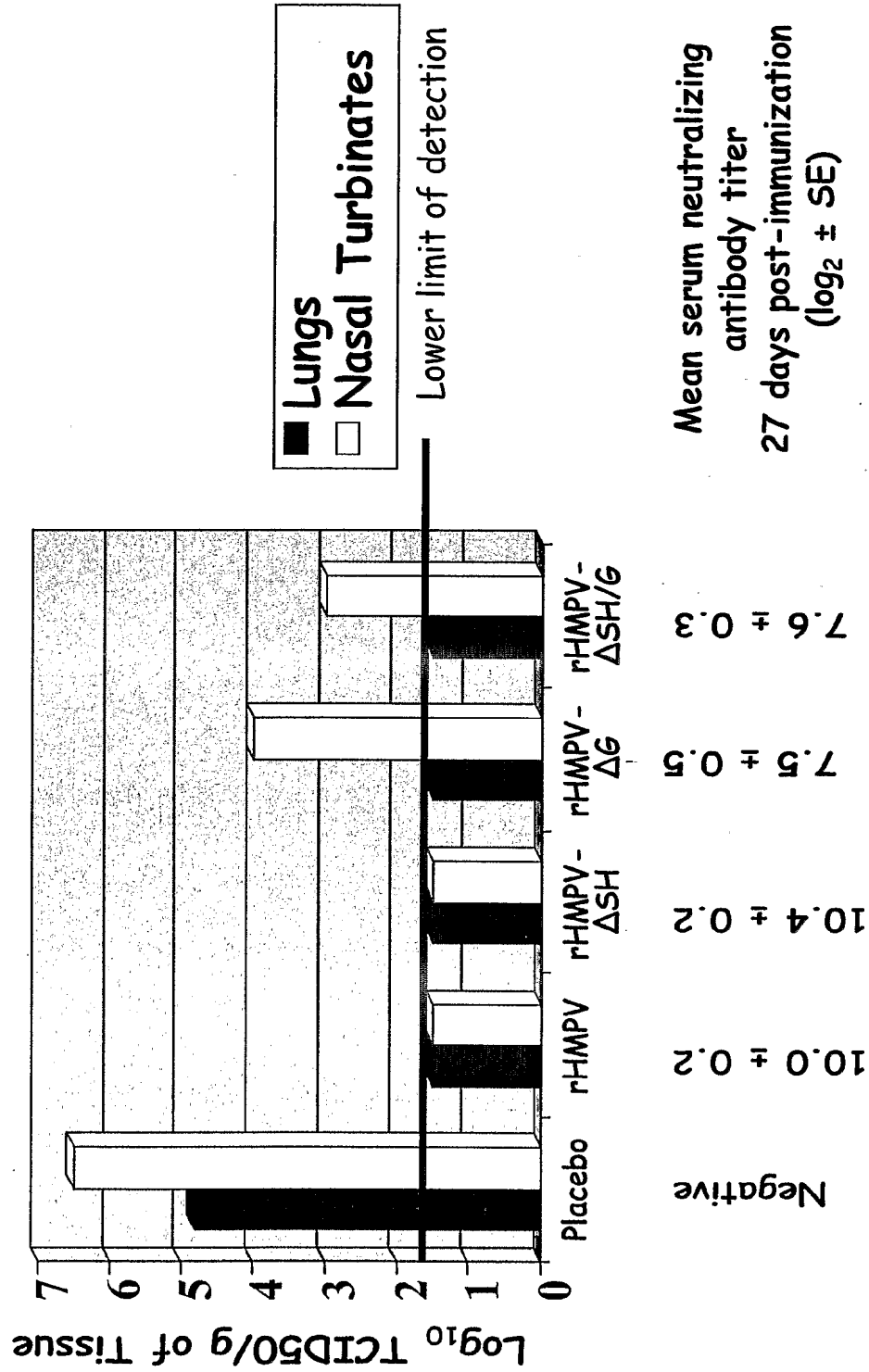


Fig. 17C

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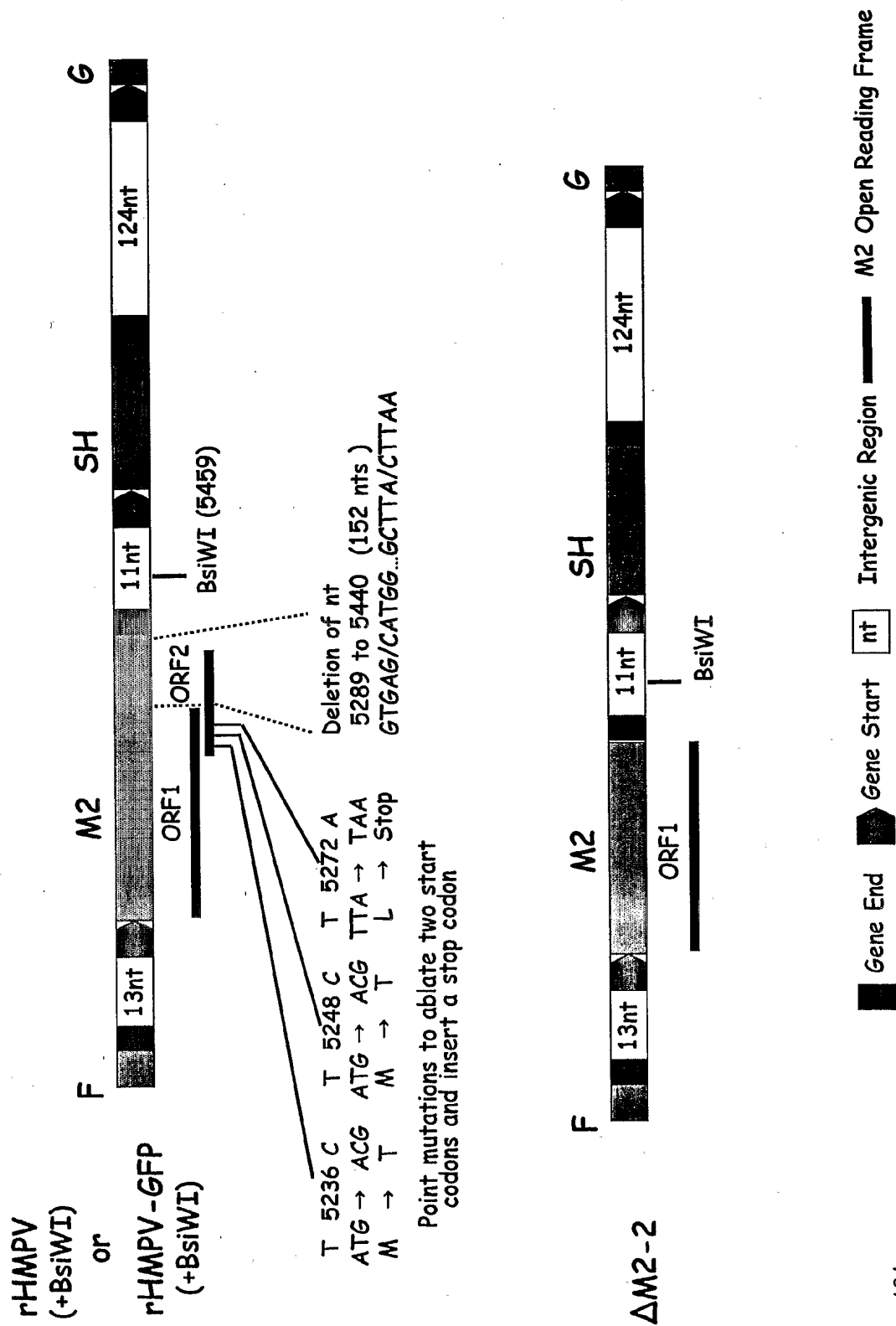


Fig. 18A

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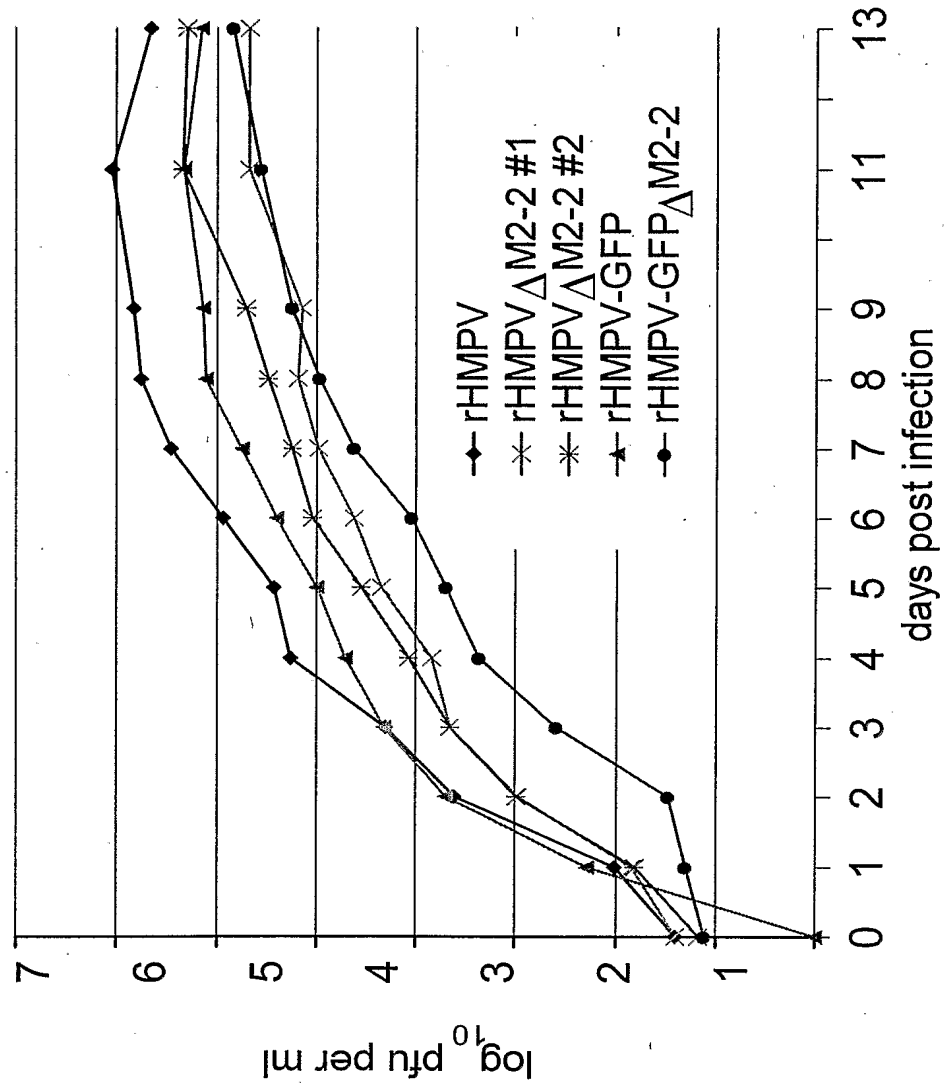


Fig. 18B

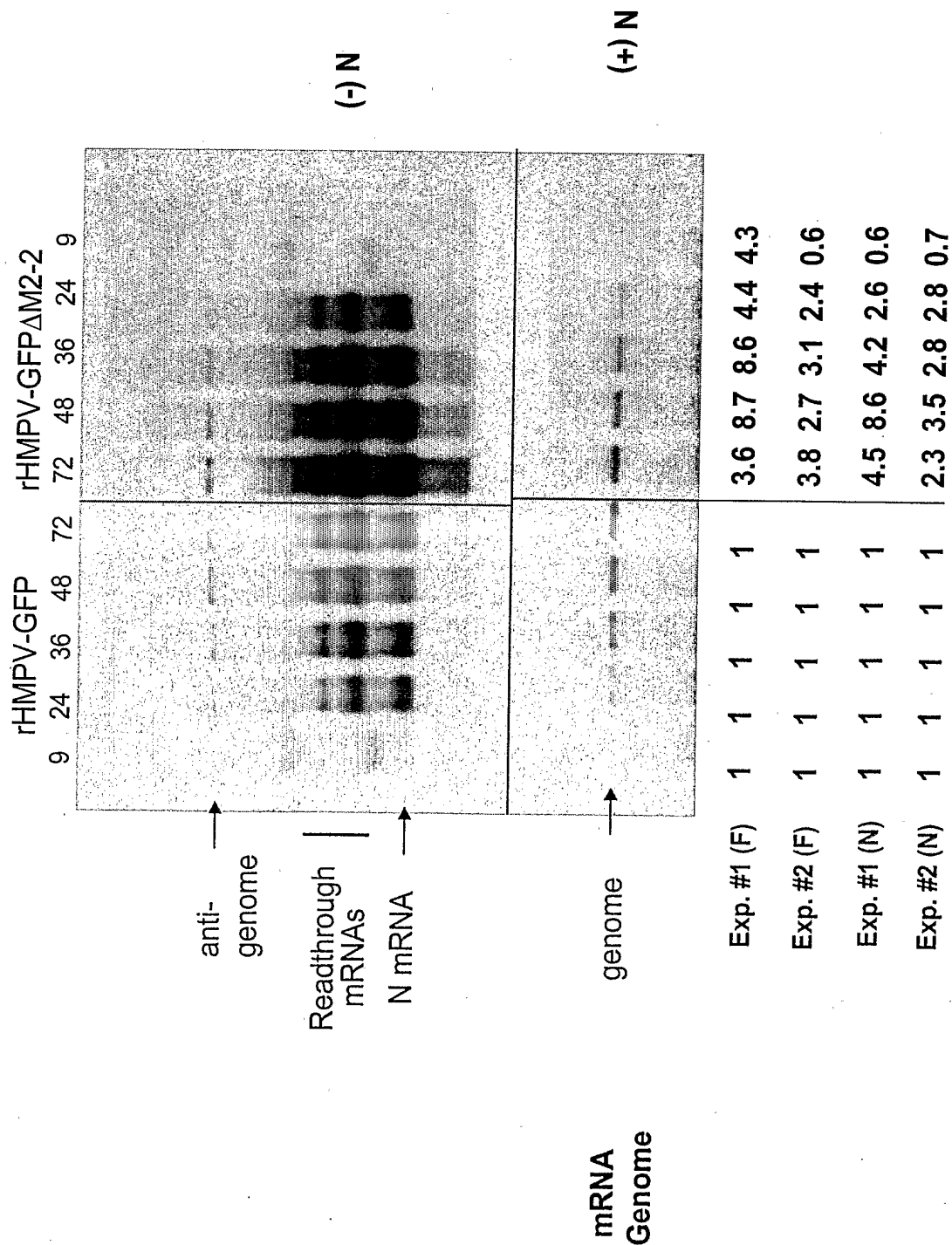


Fig. 18C

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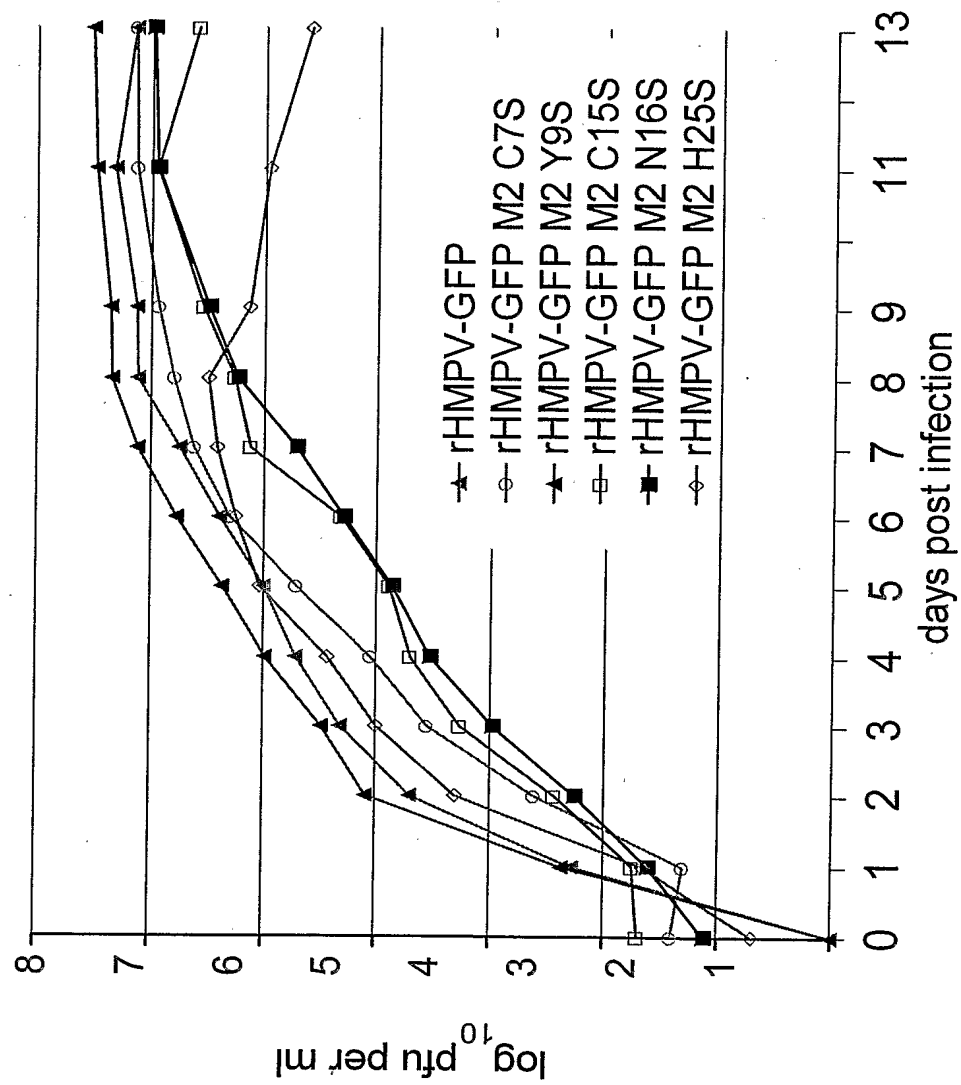


Fig. 20

4711	GGGACAAATCATAATG	TCT CGC AAG GCT CCA	TGC AAA TAT	GAA GTG CGG GGC AAA	TGC AAC AGA	4775
HMPV						
rHMPV-GFP ΔM2-1	tag	tag	t aaa	t aa		
HMPV	M	S R K A P C K Y E V R G K C N R				
rHMPV-GFP ΔM2-1	*	*	N K	N N		

Fig. 21

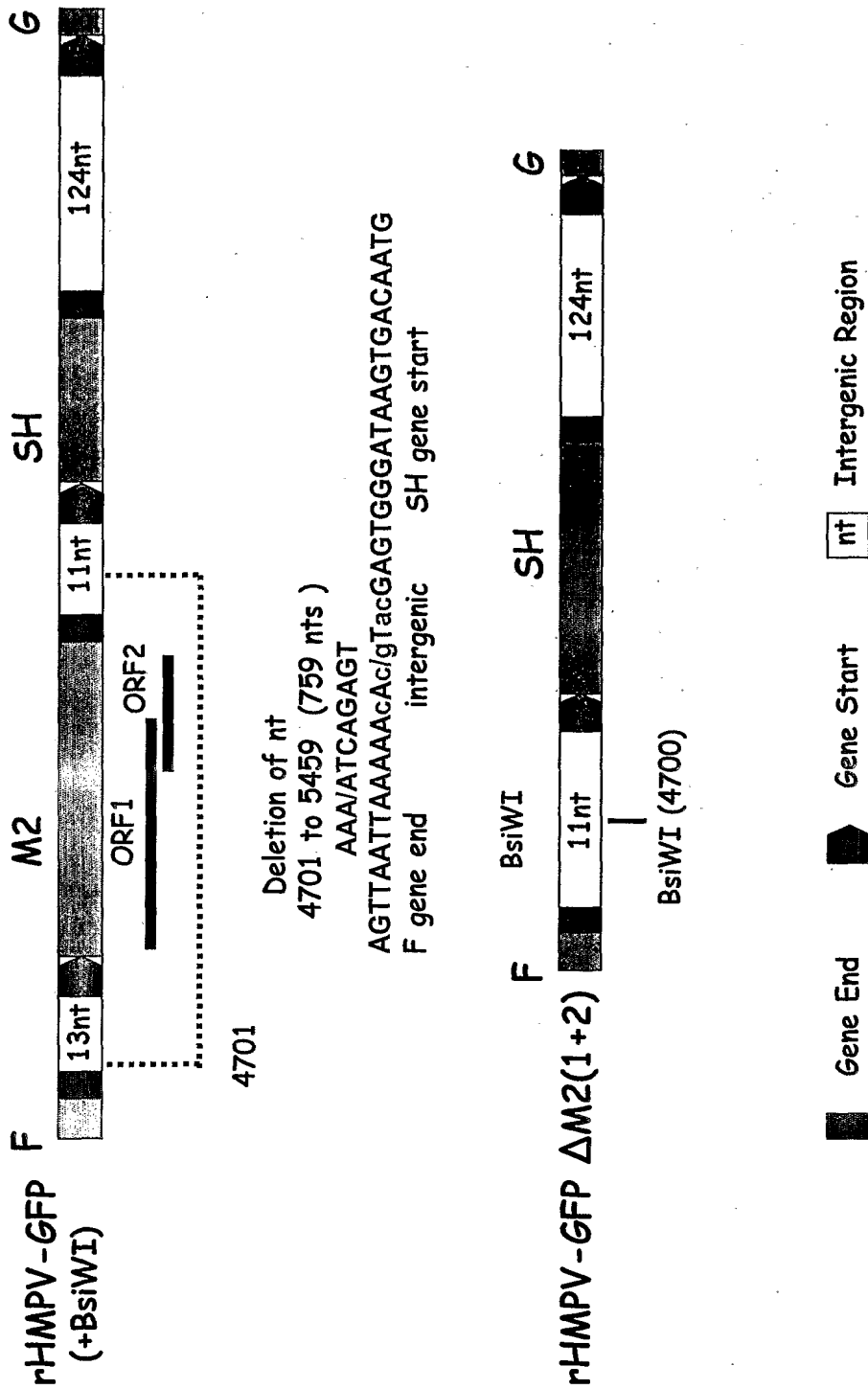


Fig. 22A

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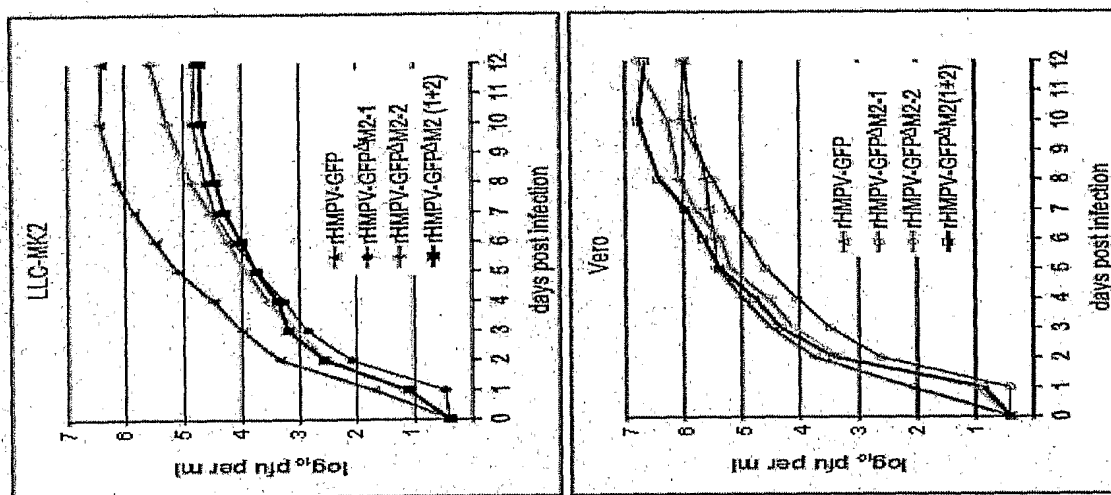


Fig. 22B

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Reduction of virus yield in presence of exogenous interferon (IFN) type I

Virus	MOI	Fold reduction of virus yield			
		10 U IFN	100 U IFN	1000 U IFN	
rHMPV-GFP	1.0	5	160	1680	
rHMPV-GFP Δ M2(1+2)	1.0	19	1130	no virus	
rHMPV-GFP Δ M2-2	1.0	13	250	no virus	
RSV-GFP	0.01	4	17	90	

Fig. 22C

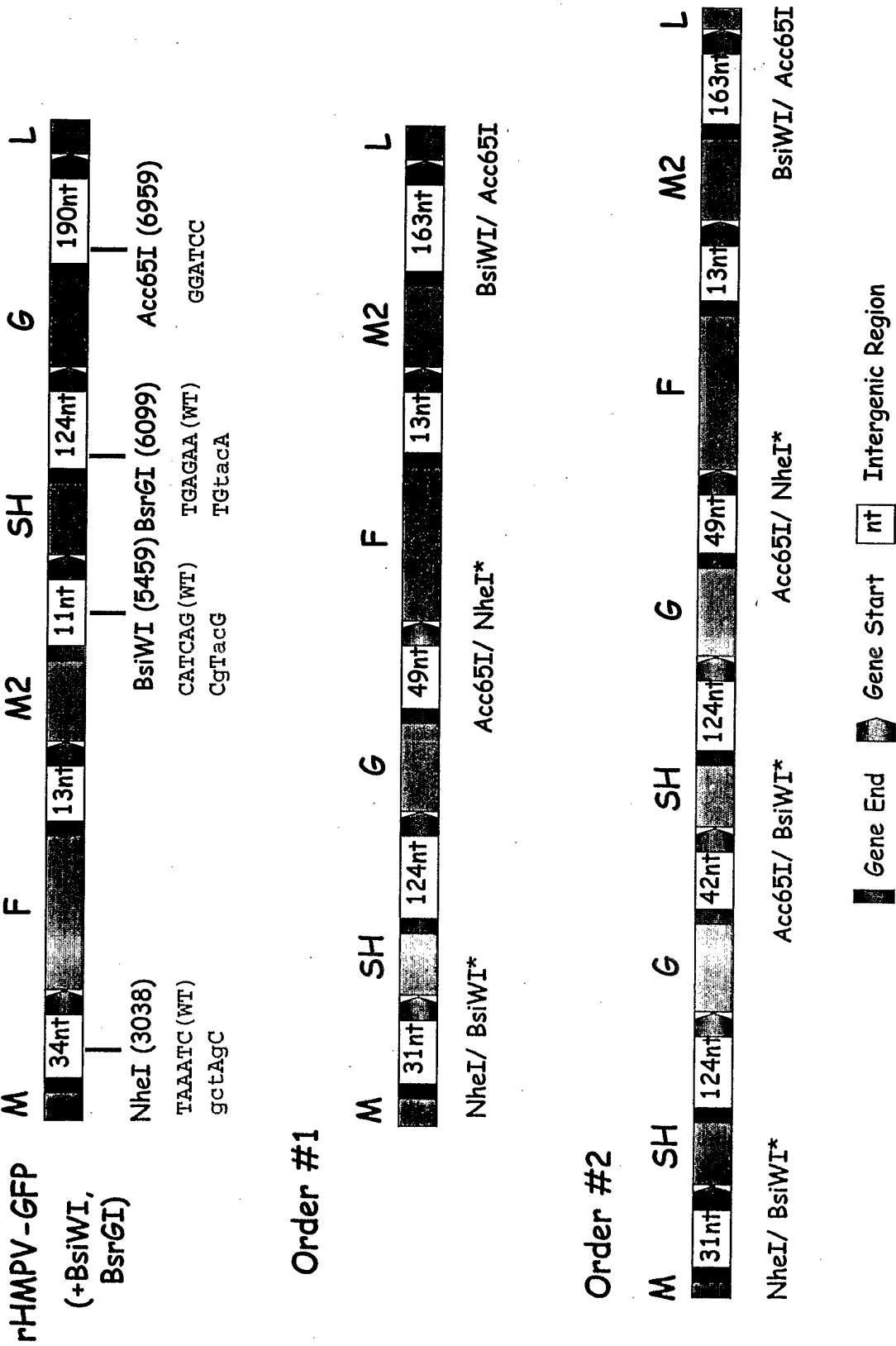


Fig. 23A

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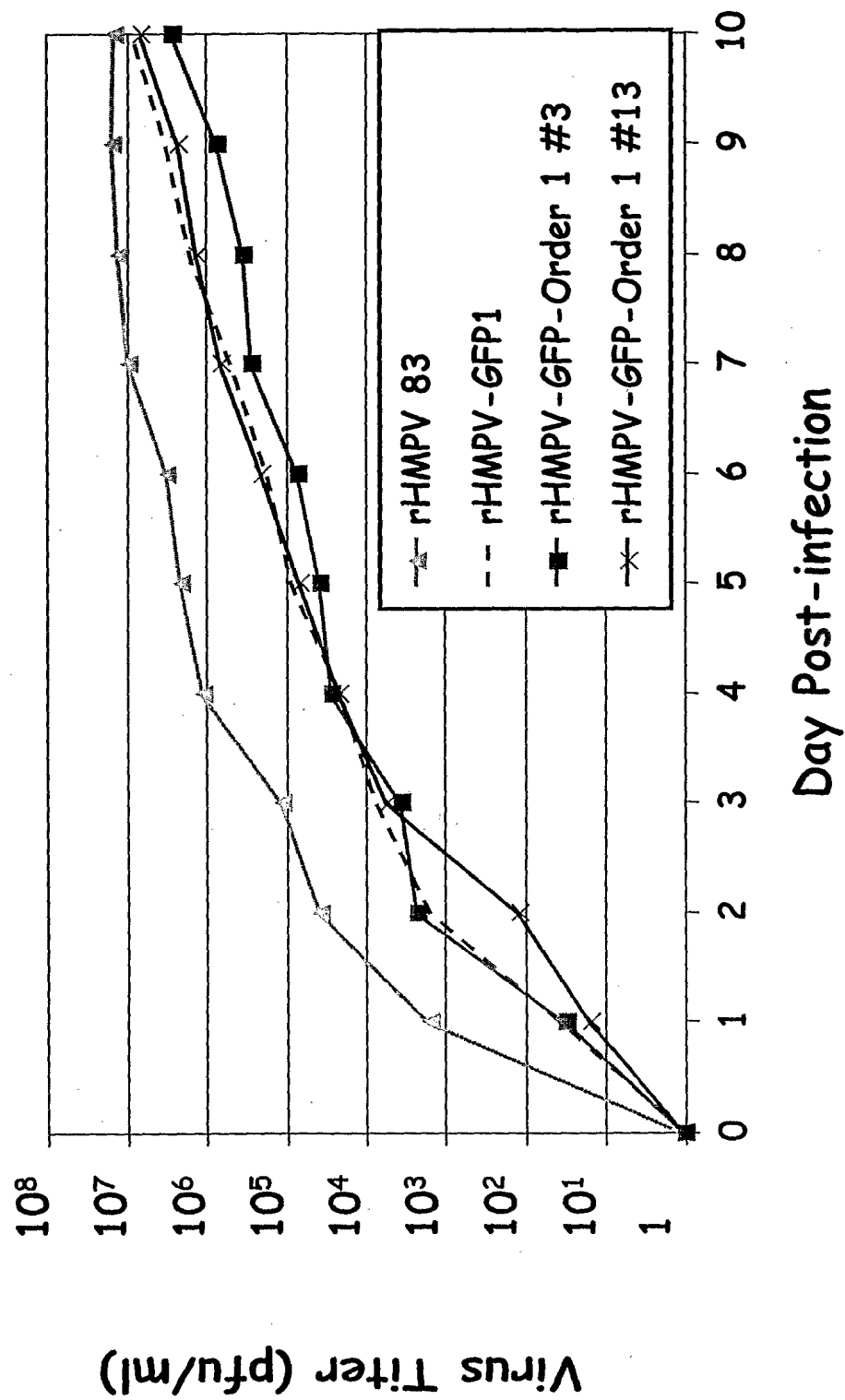


Fig. 23B

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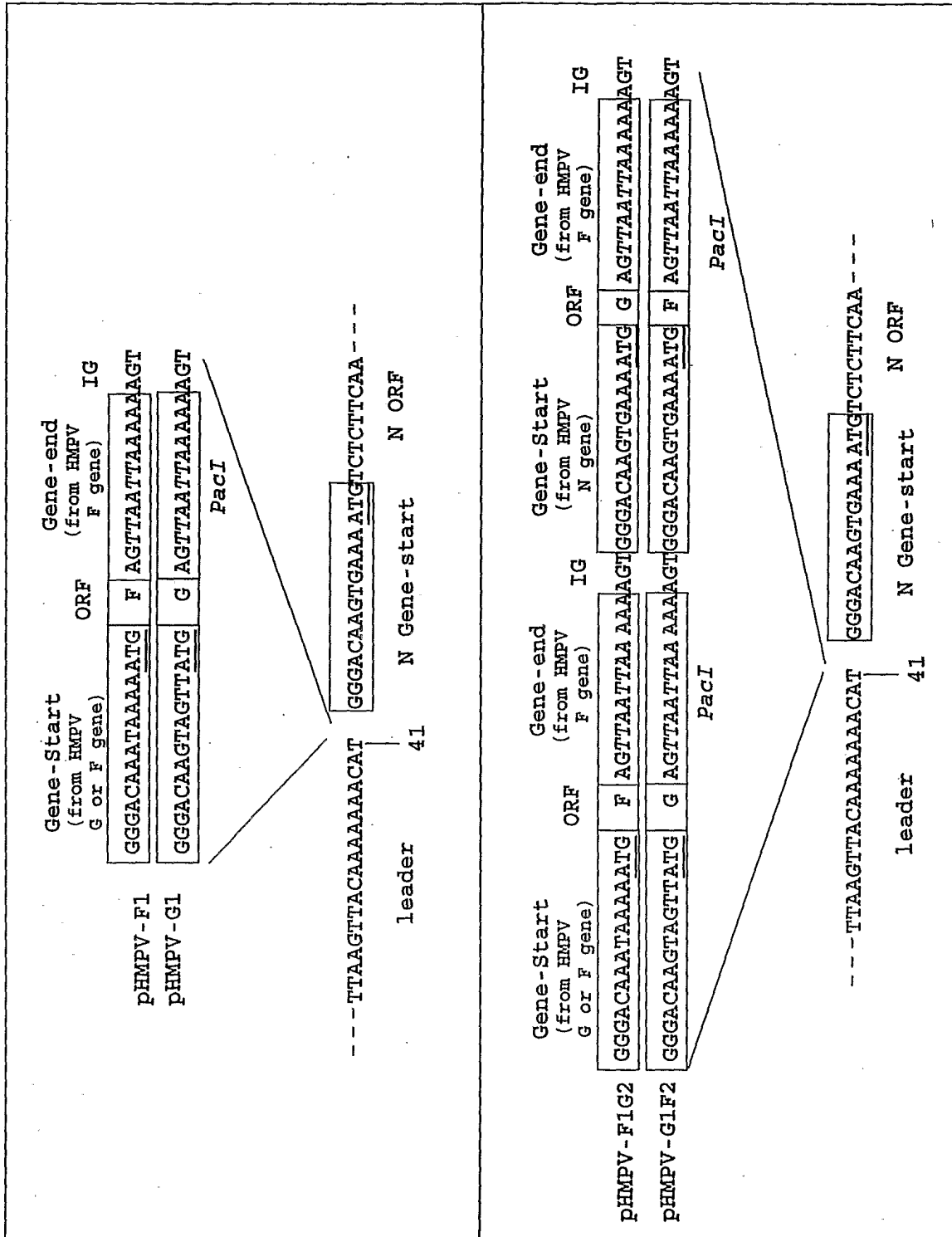


Fig. 24A

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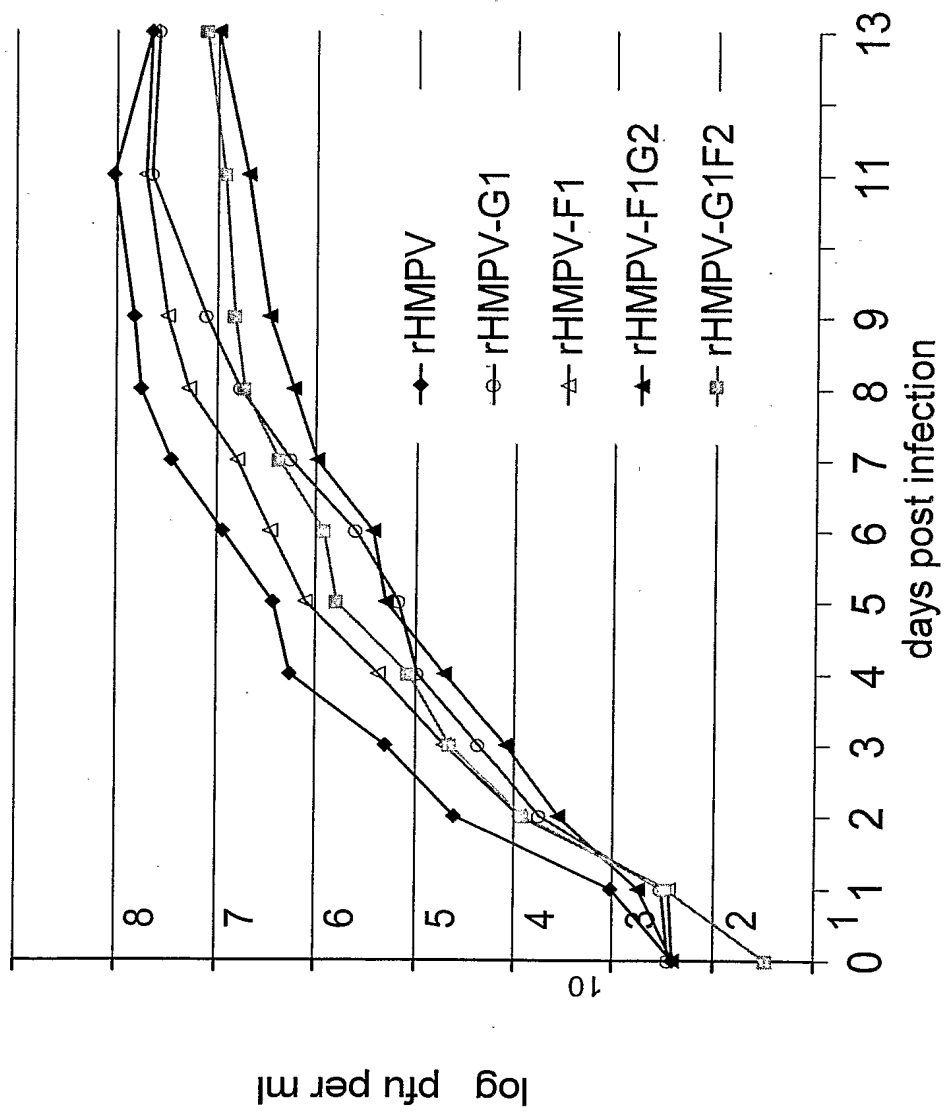


Fig. 24B

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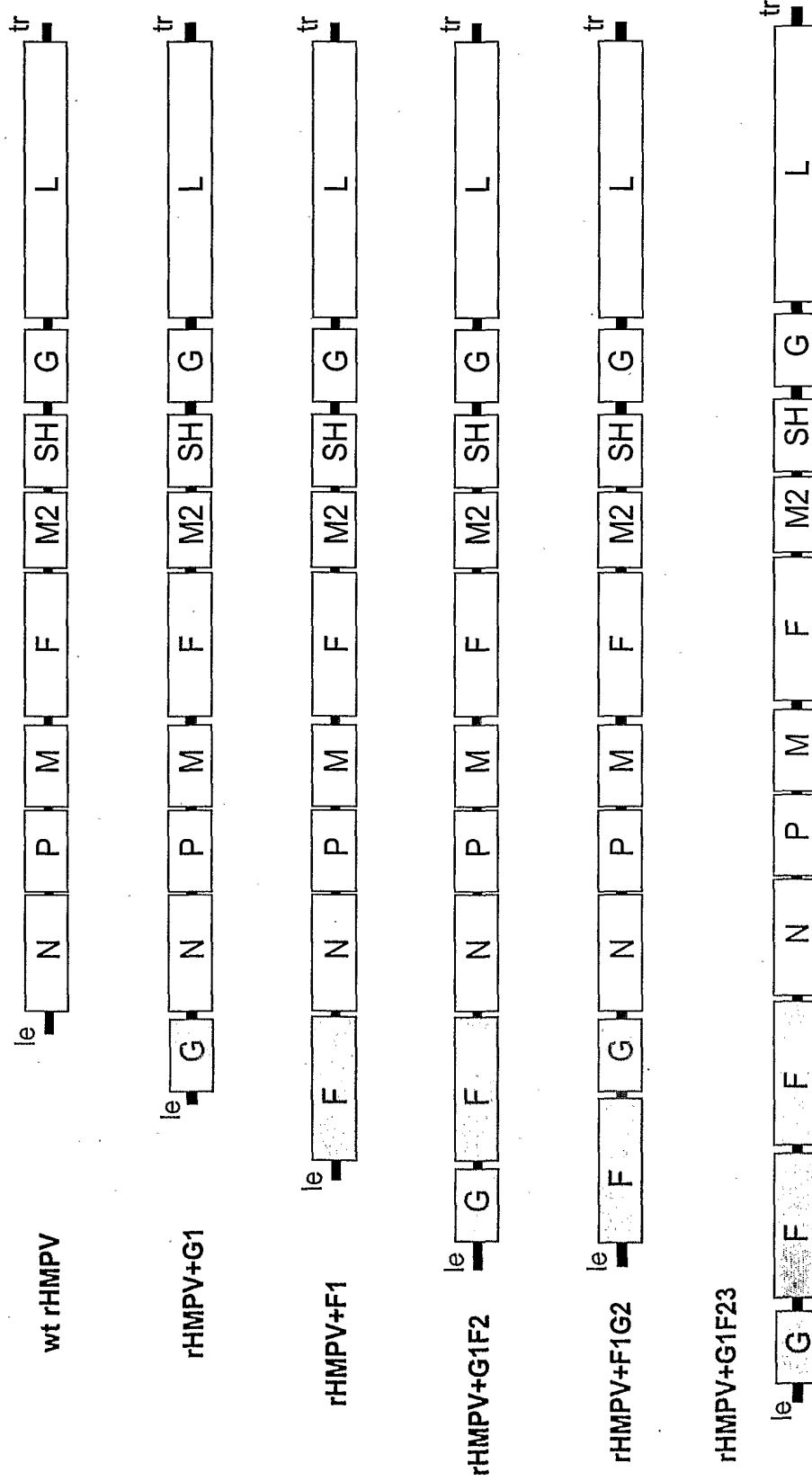


Fig. 25A

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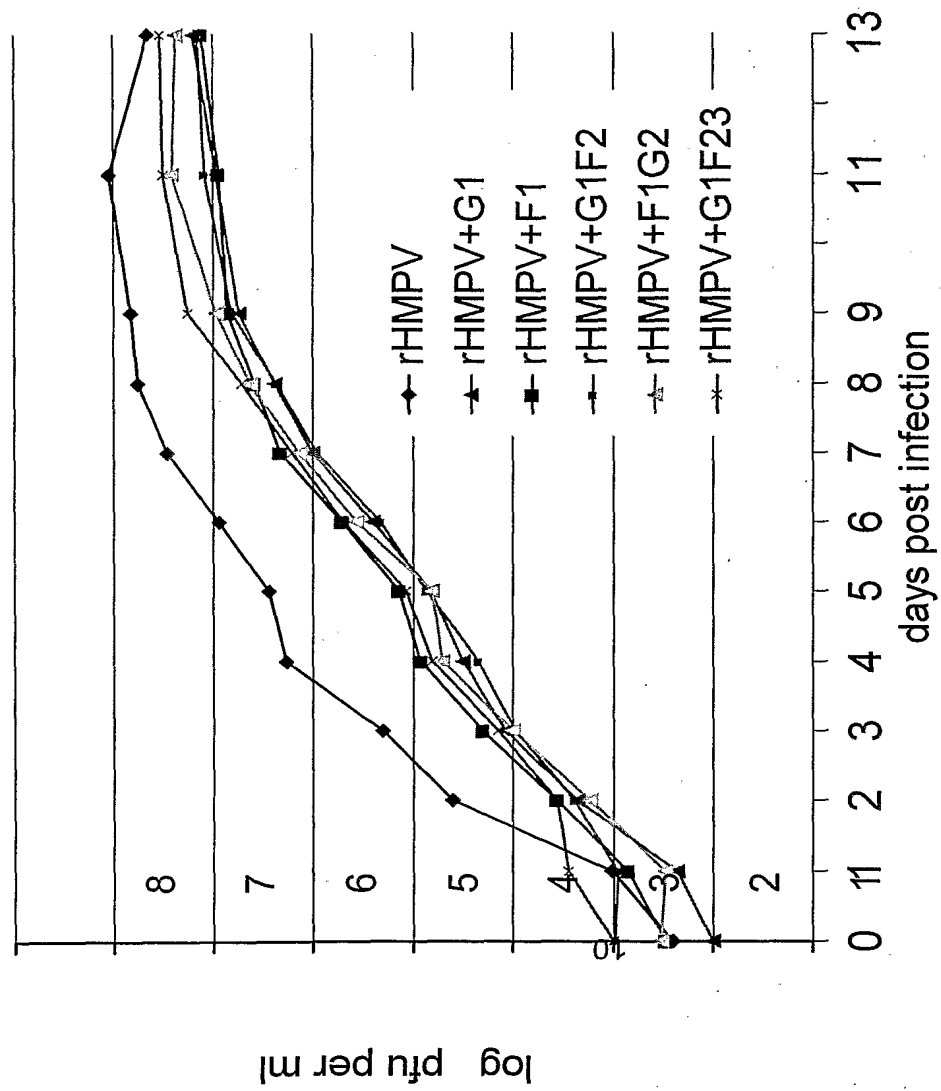


Fig. 25B

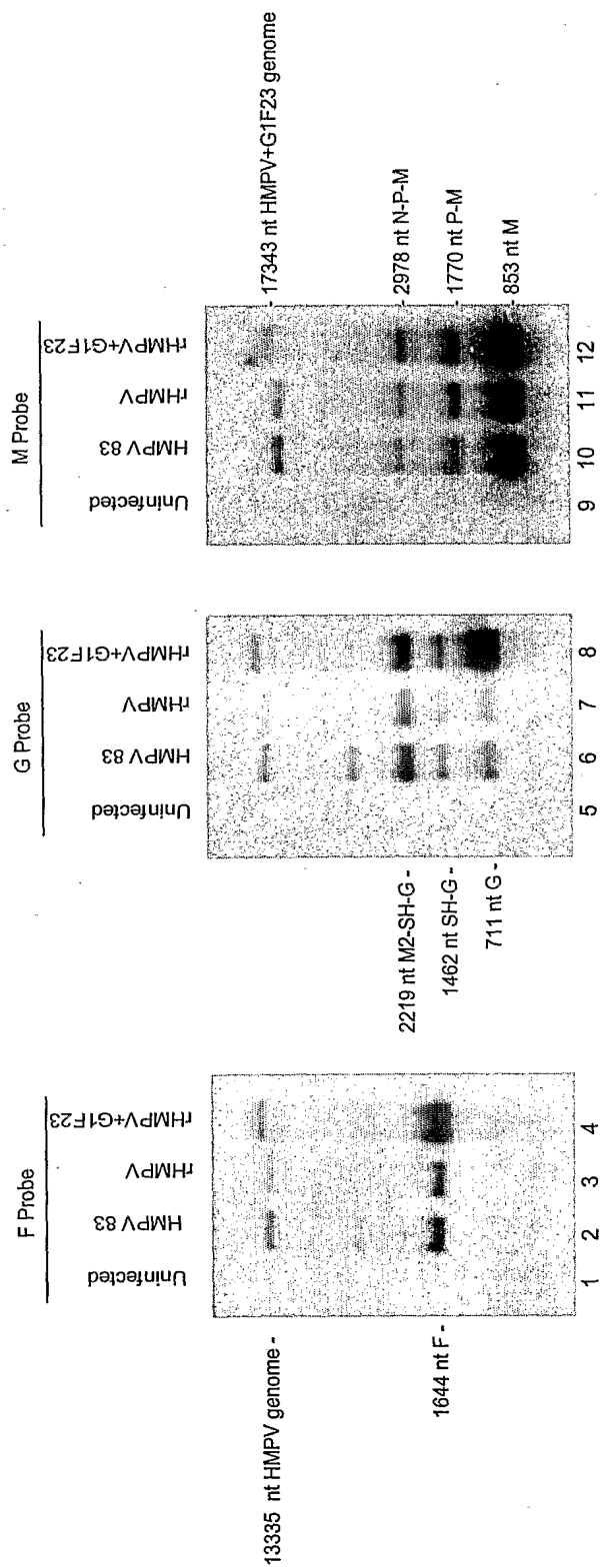


Fig. 25C

RSV A2 Mutations F521L and A48

	F521L (RSV)		
--	-------------	--	--

456

hRSV A2 hPIV3 hPIV1 hMPV 83 hMPV OOI	507 442 442 442 442	E L T E R D L I V L S G L R F Y R E F R L P K K V D L E M I I N D K A I S P P K N L I W L - S F P R A E N A V D Y Y Q S F I G I K F F L K F I E P Q L D E D L T I Y M K D K A L S P P K K - S N W D T V Y P P A A E C A V D N Y S S F I G F K F F L K F I E P Q L D E D L T I Y M K D K A L S P P R K - A A W D S V Y P P D K E L S E Q D F L E L A A I Q F F E Q E F S V P P E K T N L E M V L N D K A I S P P K R L I W S - V Y P P K K E L S E Q D F L E L A A I Q F F E Q E F S V P P E K T N L E M V L N D K A I S P P K R L I W S - V Y P P K K	593 517 517 528 528	R588A D589A	A8 (double amino acid substitution)
hRSV A2 hPIV3 hPIV1 hMPV 83 hMPV OOI		N Y M P S H I Q N Y I B H E K L K F S E S D K S R R V L E Y Y L R D N K F N - - - - S N - - - L L Y R T N A S N E S R R L V E V F I A D S K F D - - - - S N - - - L Y Y K V P E S E E T R R R L I E V F I N D N N F N N Y L P E T I K N R R Y L E E T F N A S D S L K T R R R V L E Y Y L K D N K F D N Y L P E K I K N R R Y L E E T F N A S D S L K T R R R V L E Y Y L K D N K F D			

Fig. 26A

BPIV3 Mutation I1103V

I1103V

bPIV3	1087	R	V	G	I	S	R	R	G	-	G	L	T	Y	N	L	L	R	K	I	S	N	Y	D	L	V	Q	Y	E	T	L	S	1115
hPIV3	1087	R	V	G	I	N	R	R	G	-	G	L	T	Y	N	L	L	R	K	I	S	N	Y	D	L	V	Q	Y	E	T	L	S	1115
hPIV1	1087	R	A	S	V	R	R	G	-	G	L	T	S	Y	S	I	L	R	R	I	N	Y	D	L	V	Q	Y	E	T	L	S	1115	
hRSV A2	1153	K	T	S	A	I	D	L	T	D	I	D	R	A	T	E	M	M	R	K	N	I	T	L	L	I	R	I	L	P	L	1182	
hMPV 83	1078	R	T	S	A	I	N	G	E	D	I	D	R	A	V	S	M	M	L	E	N	L	G	L	L	S	R	I	L	S	V	1107	
hMPV 001	1078	R	T	S	A	I	N	G	E	D	I	D	R	A	V	S	M	M	L	E	N	L	G	L	L	S	R	I	L	S	V	1107	

HPIV3 Mutation T1558I

T1558I

hPIV3	1539	K	V	F	K	R	F	W	D	C	G	V	L	N	P	I	Y	G	P	N	T	A	S	Q	D	Q	I	K	L	A	L	S	Y	C	E	1572
hPIV1	1539	K	I	F	K	R	F	W	D	A	G	V	V	E	P	V	Y	G	P	N	L	S	N	Q	D	K	I	L	L	A	L	S	Y	C	E	1572
hRSV A2	1586	K	V	I	K	Y	I	L	S	Q	D	A	S	L	H	R	V	K	G	C	H	S	F	K	L	W	F	L	K	R	L	N	Y	A	E	1619
hMPV 83	1507	K	V	K	K	R	I	M	L	Y	D	V	K	F	L	S	L	V	G	Y	I	G	F	K	N	W	F	I	E	Q	L	R	S	A	E	1540
hMPV 001	1507	K	V	K	K	R	I	M	L	Y	D	V	K	F	L	S	L	V	G	Y	I	G	F	K	N	W	F	I	E	Q	L	R	S	A	E	1540

RSV A2 Mutation C319Y

C319Y

hPIV3	258	K	L	Q	S	M	Y	Q	K	G	N	N	L	W	E	V	I	D	K	L	F	P	I	M	G	E	K	T	F	D	V	I	288		
hPIV1	258	R	S	S	K	I	T	C	K	G	E	N	N	L	W	E	V	I	D	K	L	F	P	I	M	G	E	K	T	F	D	V	I	288	
hRSV A2	302	R	C	G	F	N	N	V	I	L	T	N	K	L	F	L	Y	G	D	C	I	L	K	I	F	H	N	E	G	F	Y	N	I	I	332
hMPV 83	239	R	S	N	L	Q	G	M	I	L	T	N	K	L	F	L	Y	G	D	C	I	L	K	I	F	H	N	E	G	F	Y	N	I	I	269
hMPV 001	239	R	S	N	L	Q	G	M	I	L	T	N	K	L	F	L	Y	G	D	C	I	L	K	I	F	H	N	E	G	F	Y	N	I	I	269

Fig. 26C

RSV A2 Mutation Q831L

Q831L

hPIV3	772	GDNQA	I	A	V	T	T	R	V	P	N	N	Y	D	Y	R	I	K	K	E	L	V	Y	K	D	V	V	R	F	F	D	S	L	R	E	V	M	D	D	L	G	H	816	
hPIV1	772	GDNQA	I	A	V	T	S	R	V	P	V	T	Q	T	Y	K	Q	K	K	T	H	V	Y	E	E	I	T	R	Y	F	F	G	A	L	R	E	V	M	F	D	L	G	H	816
hRSV A2	810	GDNQS	I	D	I	S	K	P	I	R	L	M	E	G	Q	T	-	H	A	Q	A	D	Y	L	A	L	N	S	I	K	L	L	Y	K	E	Y	A	G	I	G	H	853		
hMPV 83	744	GDNQS	I	D	V	S	K	P	V	K	L	S	E	G	L	D	-	E	V	K	A	D	Y	L	A	V	K	M	L	K	E	E	I	R	D	A	Y	R	N	I	G	H	787	
hMPV 001	744	GDNQS	I	D	V	S	K	P	V	K	L	S	E	G	L	D	-	E	V	K	A	D	Y	L	A	V	K	M	L	K	E	E	I	R	D	A	Y	R	N	I	G	H	787	

RSV A2 Mutations M1169V, D1183E and C9

M1169V

D1183E

hPIV3	1087	R	V	G	I	N	R	R	G	-	G	L	T	Y	S	L	R	K	I	S	N	Y	D	L	V	Q	Y	E	T	L	S	R	T	L	R	R	S	
hPIV1	1087	R	A	S	V	R	R	G	-	G	L	S	Y	S	I	L	R	R	L	I	N	Y	D	L	L	Q	Y	E	T	L	S	R	T	L	R	R	S	
hRSV A2	1153	K	T	S	A	I	D	L	T	D	I	D	R	A	T	E	M	M	L	E	N	Y	D	L	L	I	R	I	L	P	L	D	C	N	R	S		
hMPV 83	1078	R	T	S	A	I	N	G	E	D	I	D	R	A	V	S	M	M	L	E	N	Y	D	L	L	I	R	I	L	P	L	D	C	N	R	S		
hMPV 001	1078	R	T	S	A	I	N	G	E	D	I	D	R	A	V	S	M	M	L	E	N	Y	D	L	L	I	R	I	L	P	L	D	C	N	R	S		

hPIV3	1160	L	I	V	S	D	K	I	R	Y	E	D	M	C	S	V	D	L	A	I	A	L	R	Q	K	M	W	I	H	L	S	G	C	R	M	T	S	G	L	E	T	P	1160		
hPIV1	1160	K	P	V	K	D	N	I	E	Y	E	M	L	S	I	-	C	S	E	L	A	I	G	L	R	Q	K	M	W	F	H	L	T	Y	G	R	P	I	H	G	L	E	T	P	1160
hRSV A2	1222	D	K	R	E	I	L	S	M	E	N	L	S	I	-	C	S	E	L	S	K	Y	T	V	R	E	R	S	W	L	S	N	-	-	-	-	-	-	-	-	-	-	-	1222	
hMPV 83	1147	T	E	I	P	I	K	S	N	G	R	L	I	C	-	C	-	I	S	R	T	L	R	E	T	S	W	N	N	M	E	-	-	-	-	-	-	-	-	-	-	-	1147		
hMPV 001	1147	T	E	I	P	I	K	S	N	G	R	L	I	C	-	C	-	I	S	R	T	L	R	E	T	S	W	N	N	M	E	-	-	-	-	-	-	-	-	-	-	-	1147		

D1187A* R1189A*

K1188A* E1190A*

E1208A* R1209A*

* Six point mutations collectively designated C9

Fig. 26D

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RSV A2 Mutation N43I

hPIV3	24	I	V	K	G	K	I	A	Q	L	H	T	I	M	S	L	P	Q	P	Y	D	M	D	D	S	I	L	V	I	52	
hPIV1	24	I	V	K	S	K	I	A	Q	L	H	V	L	L	D	I	N	Q	Q	Y	D	L	K	D	N	S	I	I	N	I	52
hRSV A2	23	I	S	F	S	E	C	N	A	L	G	S	Y	I	-	F	N	G	P	Y	L	K	N	D	Y	T	N	L	I	S	50
hMPV 83	22	I	S	F	S	E	T	N	A	I	G	S	C	L	-	L	K	R	P	Y	L	K	N	D	N	T	A	K	V	A	49
hMPV 001	22	I	S	F	S	E	T	N	A	I	G	S	C	L	-	L	K	R	P	Y	L	K	N	D	N	T	A	K	V	A	49

N43I

BPIV3 Mutation T1711I

T1711I

hPIV3	1690	P	E	T	I	E	D	W	D	P	I	E	D	E	N	I	L	D	N	I	V	K	T	V	N	D	N	C	S	D	N	Q	K	R	N	K	S	S	Y	F	W	1729	
hPIV3	1690	P	E	V	I	D	D	W	D	P	I	E	D	E	N	M	L	D	N	I	V	K	T	I	N	D	N	C	C	N	K	D	N	K	R	N	K	I	N	N	F	W	1729
hPIV1	1690	P	E	V	L	E	D	W	D	P	I	E	D	E	N	M	L	D	N	I	V	K	T	I	N	D	N	C	C	N	K	D	N	K	R	N	K	I	N	N	F	W	1727
hRSV A2	1744	P	L	L	S	N	K	K	L	I	K	S	S	A	M	I	R	T	N	Y	S	K	Q	L	Y	N	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	1772
hMPV 83	1608	P	M	V	N	-	-	-	-	-	-	-	-	-	-	L	T	Q	V	I	D	P	T	L	D	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	1626
hMPV 001	1608	P	M	V	N	-	-	-	-	-	-	-	-	-	-	L	T	Q	V	I	D	P	T	L	D	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	1626

Fig. 26F

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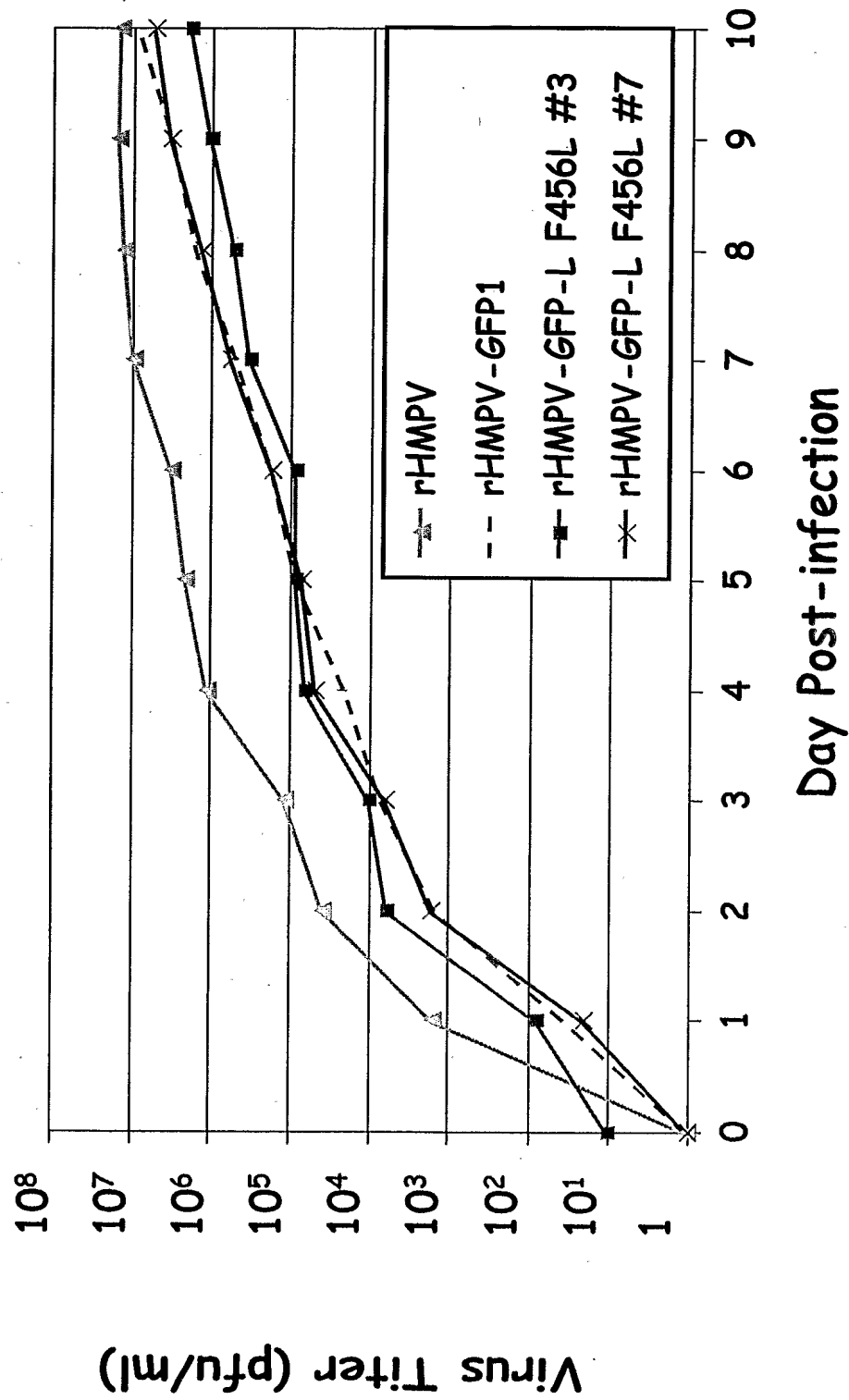


Fig. 27

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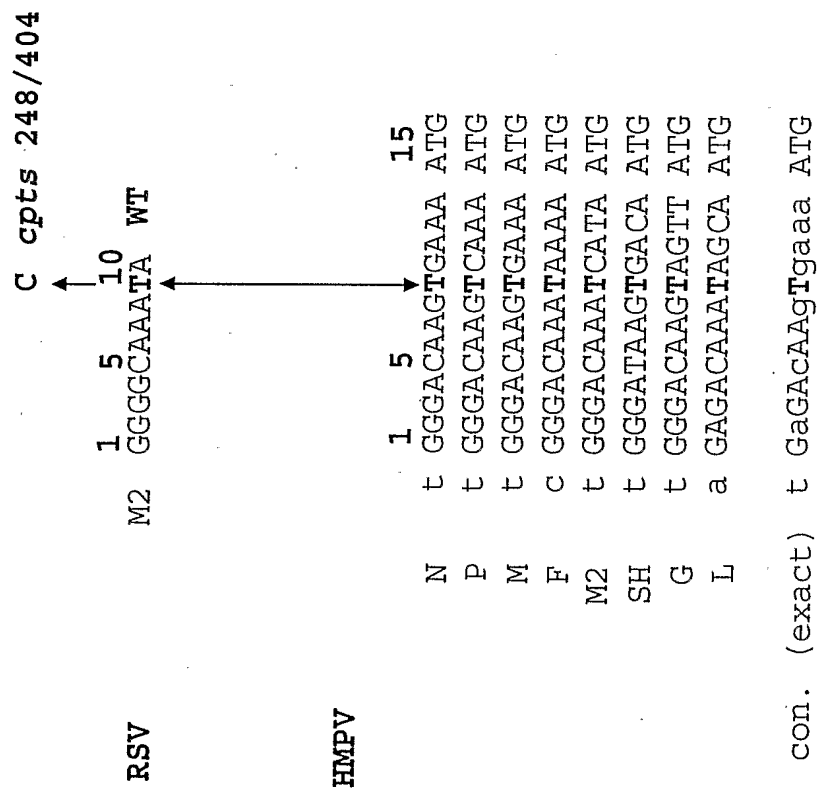
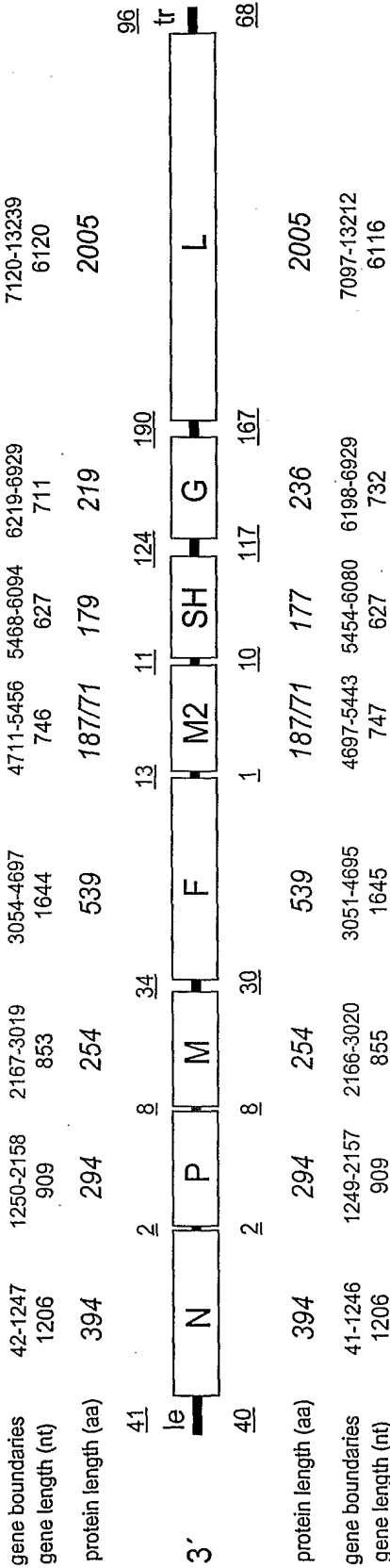


Fig. 28

CAN97-83, 13,335 nucleotides



CAN98-75, 13,280 nucleotides

Fig. 29

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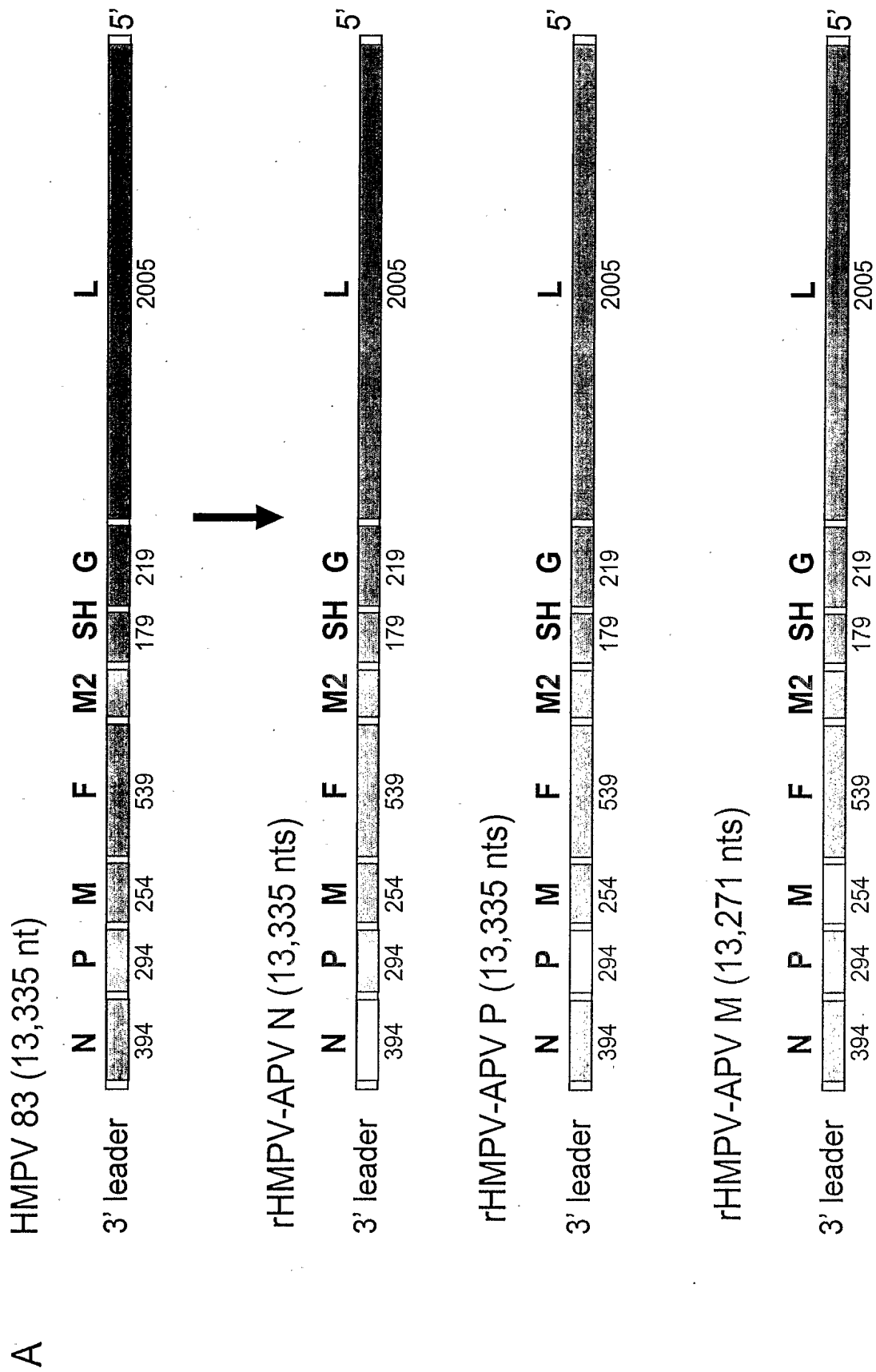


Fig. 30A

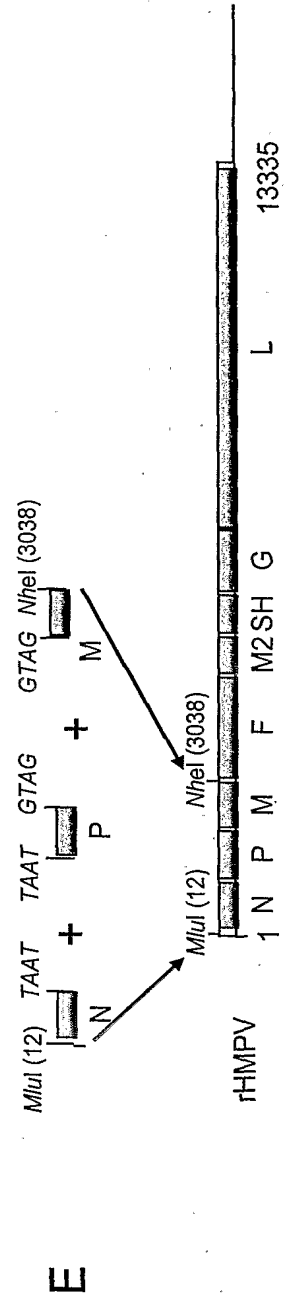
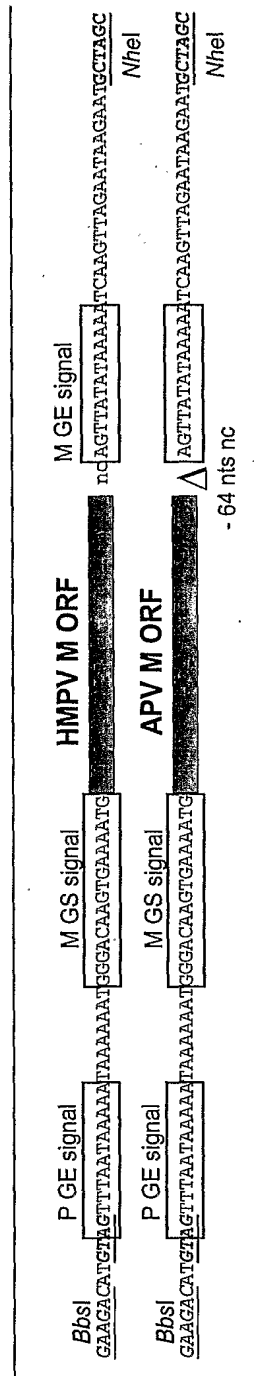
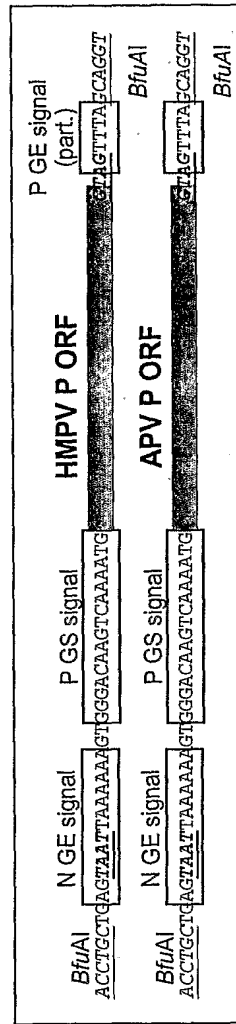
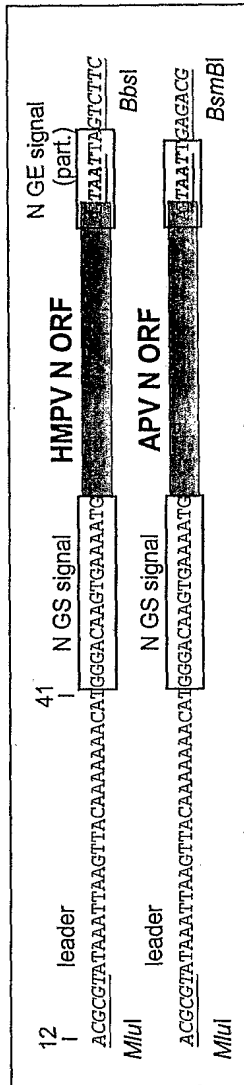


Fig. 30B-E

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	gene-end	intergenic	gene-start
	<u>1</u> <u>13</u>		<u>1</u> <u>16</u>
le (83)	aattaAGTTAcaaaaaacat		GGGACAAgTgAaaATGtctct N (83)
(75)	aattaAaTTccaaCAAAAAC-		GGGACAAaTaAaaATGtctct (75)
N (83)	ttatgAGTAAttaAAAA--	gt	GGGACAAgTcaaaATGtcatt P (83)
(75)	ttatgAGTAAttaAAAA--	ct	GGGACAAgTcaaaATGtcatt (75)
P (83)	tatgTAGTTtaataAAAA--	taaaaaat	GGGACAAgTgAaaATGgagtc M (83)
(75)	catgTAGTTtaataAAAA--	taaacaat	GGGACAAgTcaagATGgagtc (75)
M (83)	attttAGTTAtataAAAA--	tcaag- 24 nt-agaac	GGGACAAaTaAaaATGtcttg F (83)
(75)	attatAGTTAtataAAAAAA	tttag- 20 nt-aaagc	GGGACAAgTaAaaATGtcttg (75)
F (83)	cagttAGTTAattAAAA--	taaaaataaaattt	GGGACAAaTcAtAAATGtctcg M2 (83)
(75)	tagttAGTTAattAAAAAA-	t	GGGACAAaTcAtcATGtctcg (75)
M2 (83)	acttaAGTTAgtaAAAA--	cacatcagagt	GGGATAAGTgACAAATGataac SH (83)
(75)	acttaAGTTAgtaAAAA--	taaatagaat	GGGATAAaTgACAAATGaaaaac (75)
SH (83)	agtttAGTTAttttAAAA--	tattt-114 nt-aatat	GGGACAAgTagttATGgaggt G (83)
(75)	agtctAGTTAttttAAAA--	ctcta-107 nt-aaaat	GGGACAAgTggccATGgaagc (75)
G (83)	aaattAGTTAacaAAAA--	tacga-180 nt-tccaa	GaGACAAaTagcAAATGgatcc L (83)
(75)	caagtAGTTAacaAAAAAA-	ctata-157 nt-ttcaa	GGGACAAaTaacaAAATGgatcc (75)
L (83)	atgatAGTTAattAAAA--	ttaaa-91 nt	
(75)	ccattAGTTAattAAAA--	ttata-63 nt	tr (83)
consensus	AGTTAnnnAAAAA		(75)
			GGGACAAaTnnnnAATG

Fig. 31

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Percent amino acid or nucleotide sequence identity between the indicated strains of HMPV or RSV for the indicated proteins and ORFs

Viruses compared	percent amino acid sequence identity for indicated protein (percent nucleotide sequence identity for indicated ORF)									
	N	P	M	F	M2-1	M2-2	SH	G	L	
CAN97-83 vs CAN98-75	96 (84)	85 (81)	97 (85)	95 (84)	96 (85)	89 (87)	59 (69)	37 (59)	94 (84)	
CAN97-83 vs 00-1	99 (94)	95 (92)	99 (94)	98 (94)	98 (94)	96 (95)	85 (88)	70 (77)	99 (95)	
RSV A2 vs RSV B1	95 (85)	90 (85)	91 (85)	89 (81)	91 (84)	61 (69)	72 (77)	55 (67)	92 (85)	

Fig. 32

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A. SH protein

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CAN98-75 1 K E NQ K L S PT S V V T 54
CAN97-83 1 MITLDVIKSGSKTCTHLKKIKDHSCKVLLALXLLALGFFTTITINYIK 54
00-1 1 V L V 54
* ***** **..***** ***** * ***** *.***.*** *
CAN98-75 55 A L N K TKL T IRPIP LNAV L KH N TKD 108
CAN97-83 55 VENNLIQCSKTESDKEDSPSNTTSVTTKTDLTDHDITQYFKRLIORYTDSVIN- 107
00-1 55 K SS N SL TN A - 107
***** ** *.***** * ***** * ** * ****. * *
CAN98-75 109 R R HT KI GSMN TNTD EEP V DK TMT KHRKA 162
CAN97-83 108 KDTCKISRNOCTNITTYKFLCFKPEDSKINSCDRLTDLCKNKSAAEAYHTV 161
00-1 108 S N S T T N K P P VGV I 161
****. * ***** ***** . . * * * * *
CAN98-75 163 RPH T W HYL----- 177
CAN97-83 162 ECHCIYTIWKCYYHHSID---- 179
00-1 162 VK YPT ETQS 183
*** * * * *

```

Fig. 33A

B. G protein

CAN98-75	1	AR	F	KM	IRS	HR	T	S	AP	M	TL	DHA	54
CAN97-83	1	MEVKVENIR	AI	DML	KAR	VKNR	VARSKCFK	NAS	L	I	G	I	54
00-1	1	T						V				K	54
		**	*****	****	****	***	***	***	***	***	***	***	
CAN98-75	55	TS	NMTKV	CVNM	V	PS	KTPMT	AAD	NTK	P	QA	LT	108
CAN97-83	55	IQT	SESEHHT	TSSP	P	TES	KNKEAST	I	STD	NP	DIN	PN	108
00-1	55	M	NT		S	M	SR	TP	VP	S	T	SSP	108
		**	**	*	*	*	*	*	*	*	*	*	*
CAN98-75	109	LEDHLH	G	TTP	A	V	SQQT	EH	TLLR	STNR	Q	TQ	162
CAN97-83	109	SVP	SETEP	ASTP	DT	TNRL	SSVDR	STAQ	P	SESR	TKTK	PTV	162
00-1	109	A	SP	T		PPF	TH	TP	A	S	A	K	162
		*	*	*	*	*	*	*	*	*	*	*	*
CAN98-75	163	TT	T	ST	ATQ	LTNT	NTQ	TSNG	EA	AR	R	NNA	216
CAN97-83	163	PPR	ATTKAIR	RAT	TFR	MS	STGKR	-PTT	S	VQ	SDS	STTT	215
00-1	163	RTA	T	L	T	R	-S	A	P	I	A	HKN	215
		***	*	*	*	*	*	*	*	*	*	*	*
CAN98-75	217	QHTQK	STTT	THN	DDT	SSP	SS	-					
CAN97-83	216	TMQN	-----										
00-1	216	TRIQR	KSVEAN	T	STTY	NQ	TS						

Fig. 33B

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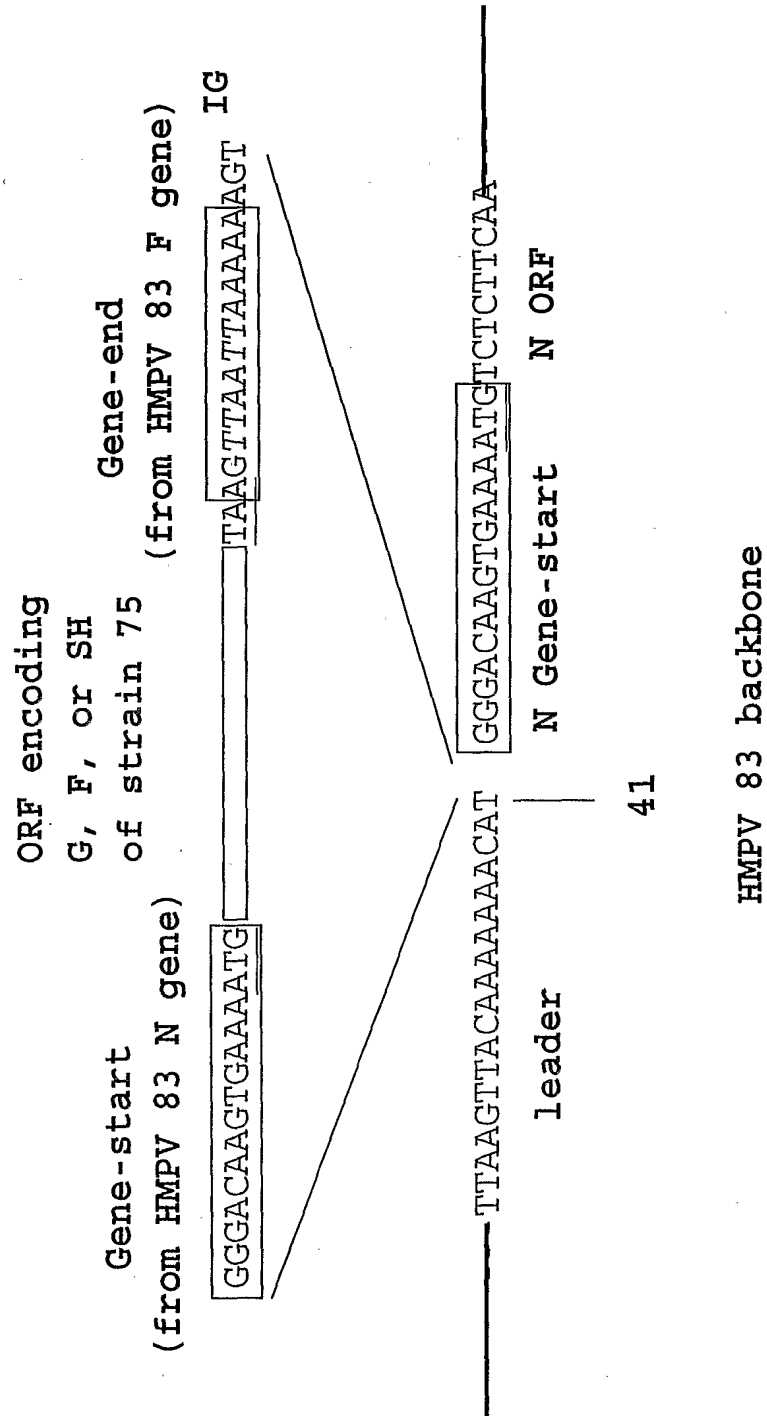


Fig. 34

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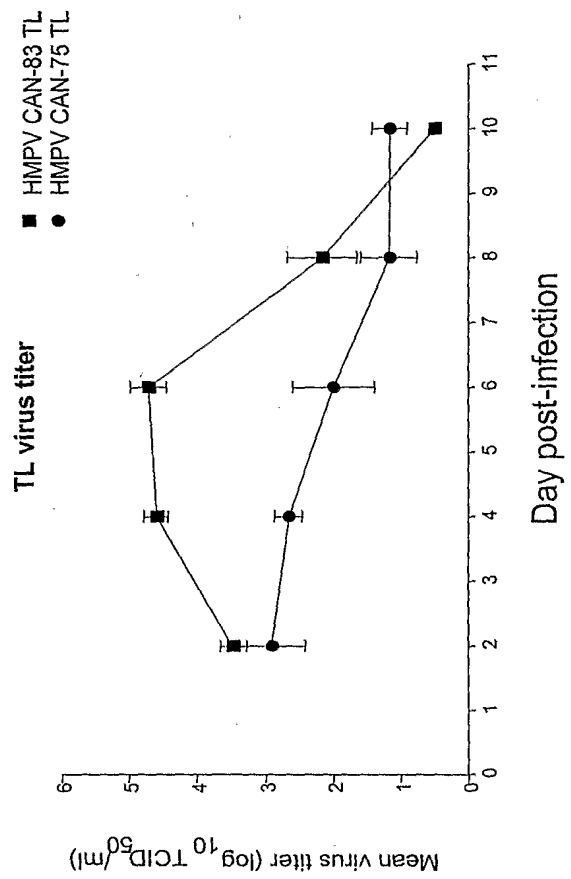
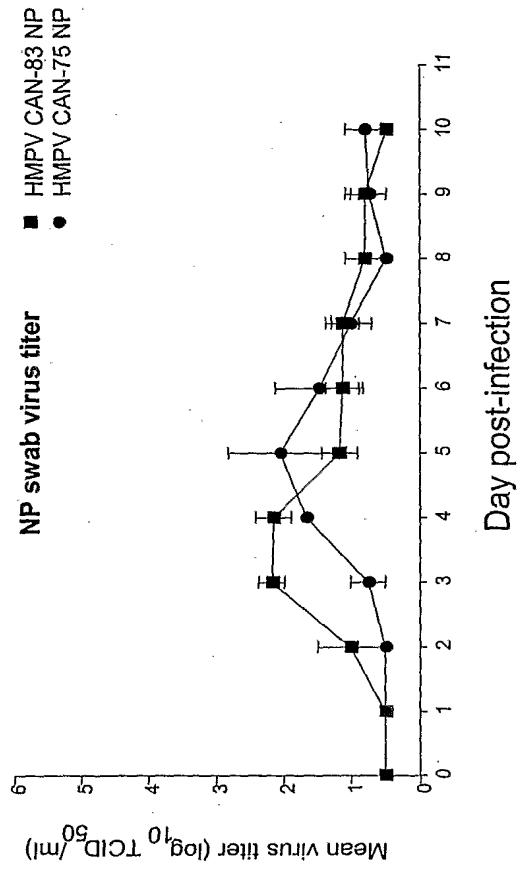


Fig. 35

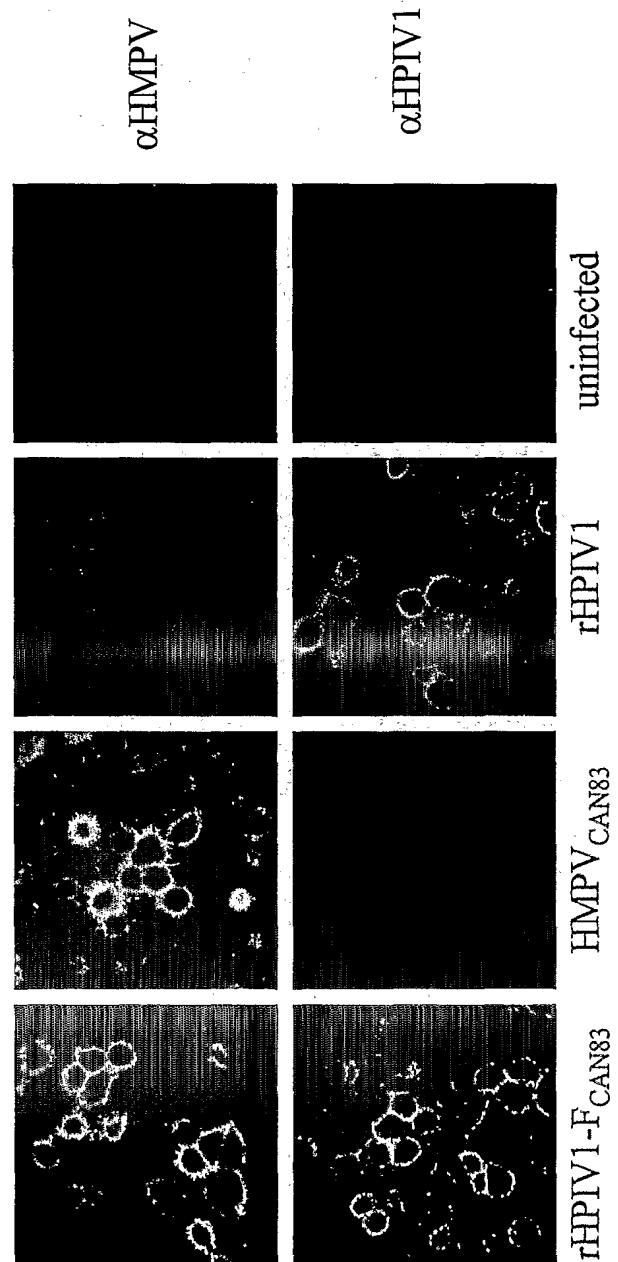
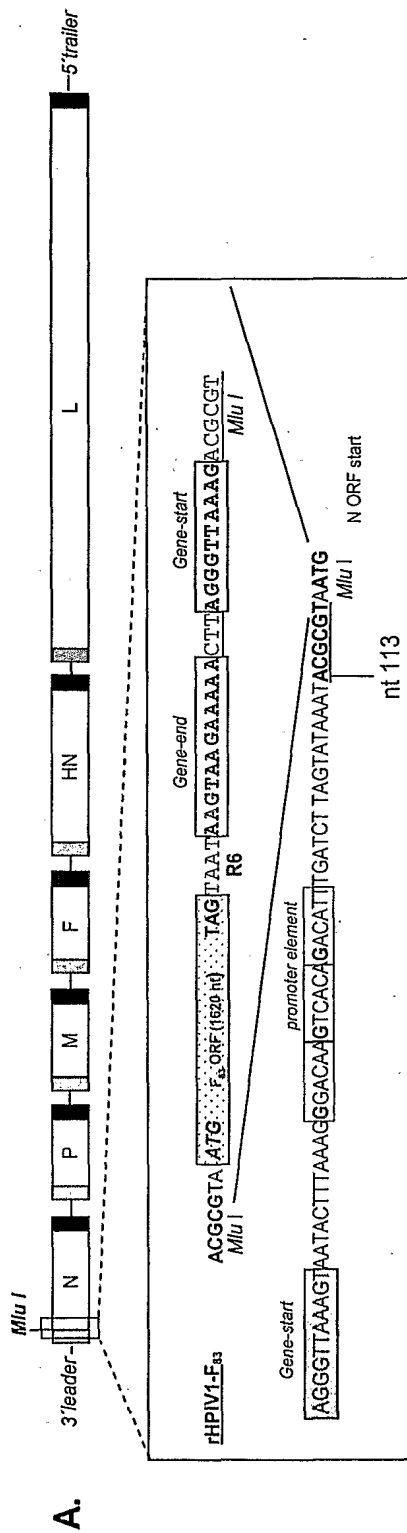


Fig. 36A & B

HMPV strain 83

	10	20	30	40	50	60	70	80	90	100
1	ACGCGAAAA	AACGCGTATA	AATTAAAGTTA	CAAAAAAACA	TGGGACAAGT	GAAAAATGTCT	CTTCAAGGGA	TTCACCTGAG	TGATCTATCA	TACAAGCATG
101	CTATATTAAA	AGAGTCTCAG	TATACAATAA	AGAGAGATGT	AGGCACAACA	ACAGCATGTA	CACCTTCATC	AGTTCAACAA	GAATAAACAC	TATTGTGTGG
201	AGAAATTTCTA	TATGCTAAGC	ATGCTGATTA	CAAAATATGCT	GCAGAAATAG	GAATACAAAT	TATTAGCACA	GCTTAGAGAT	CAGAGAGAT	ACAGCAGATT
301	CTAAGAACT	AGTCCAAGTG	AGTCCAAGTG	GTTTTAAAGT	AGAGTACTC	CTTTGGGAAA	GTTAAAAACA	ACAAAGGAGA	AGATTACAG	ATGTTAGACA
401	TACACGAGT	AGAGAAAAGC	TGGGTGGAAG	AGATAGACAA	AGAACTAAGA	AAAACAATGG	CAACTTTTGT	TAAAAGATCA	TCAGGCAATA	TTCACAAAAA
501	TCAGAGGCT	TCAGCACCCAG	ACACACTAT	AATCTTATTA	TGTGTAGGTG	CCTTAATAT	TACCAAACTA	GCAATCAACTA	TAGAAGTGG	ATTAGAGACC
601	ACAGTCAGAA	GAGCTAACCG	TGTACTAAAT	GATGCACCTCA	AAAGATACCC	TAGGATGGAC	ATACCAAAAA	TCGCTAGATC	TTTCTATGAT	TTATTTGAAC
701	AAAAAGTGA	TTACAGAAAT	TTGTTCAATG	AGTATGGCAA	AGCATTAGGC	TCATCTCTTA	CAGGCAGCAA	AGCAGAAAAT	TTATTTCGTTA	ATATATTTCAT
801	GCAAGCTTAC	GGTGTGGTTC	AAACAATGCT	GAGGTGGGGA	GTCAATTGCCA	GGTCATCTAA	CAATATAATG	TTTAGGACATG	TATCTGTCCA	AGCTGAGTTA
901	AAACAAGTCA	CAGAAAGTCTA	TGACCTGGTG	CGAGAAAATGG	GCCCTGAATC	TGGGCTCCTA	CATTTAAGGC	AAAGCCCAAA	AGCTGGACTG	TTATCACTAG
1001	CCAATTGTCC	CAACTTTTGA	AGTGTGTTTC	TCGGCAATGC	CTCAGGCTTA	GGCATAATAG	GTATGTATCG	CGGAGAGATG	CCAAACACAG	AACTATTTTC
1101	AGCAGCAGAA	AGCTATGCCA	AGAGTTTGAA	AGAAAGCAAT	AAAATTAAC	TTTCTTCTAT	AGGACTCACA	GATGAAGAAA	AGAGGCTGC	AGAACACTTT
1201	CTAAATTTGA	GTGACGACAG	TCAAAATGAT	TATGAGTAA	TAAAAAAGTG	GGACAAATCA	AAATGTCTAT	CCCTGAAGGA	AAAGATATTC	TTTTTCATGG
1301	TAAATGAAGC	GCAAAATTTGG	CAGAAAGCTTT	CCAAAAATCA	TTAAAGAAAAC	CTAGTCATAA	AAGATCTCAA	TCTATTATAG	GAGAAAAAGT	GAACACTGTA
1401	TCTGAACAT	TGGAATTACC	TACTATCAGT	AGACCTACCA	AACGACCCAT	ATTGTCCAGAG	CCGAAGTTAG	CATGGACAGA	CAAAAGTGGG	GCAATCAAAA
1501	CTGAAGCAG	GCAAAACAATC	AAAGTTATGG	ATCCTATTGA	AGAAAGAG	TTTACTGAGA	AAAGGTGCT	GCCTCCAGT	GATGGGAAA	CTCCTGCAGA
1601	AAAGAAGTTG	AAACCATCAA	CCAAATCTAA	AAAGAAGTTC	TCATTATCAC	CAATGAAC	AGGAAATAC	ACAAAGTTGG	AGAAAGATGC	TCTAGACTTG
1701	CTTTCAGACA	ATGAAGAAGA	AGATGCAGAA	TCCTCAATCT	TAACTTCTGA	AGAAAGAGAT	ACTTCATCAT	TAGACATTTGA	AGCCAGACTA	GAATCGATTG
1801	AGGAGAAAT	AAGCATGATA	TTAGGCTAT	TAAAGAACAT	CAACATTGCT	ACAGCAGGAC	CCACAGCAGC	AAGAGATGGG	ATCAGAGATG	CAATGATTGG
1901	CATAAGGGAG	GAATTAATAG	CAGACAATAT	AAAAGAAGCC	AAGGAAAAAG	CAGCAAGAAAT	GATGGAAGAA	GAATGAACC	AGCGACAAA	AATAGGAAAC
2001	GGTAGCTGAA	AATTAACTGA	AAAGGCAAG	GAGCTCAACA	AAATGTTGA	AGACAGAGC	ACAAAGTGTG	AATCCGAGAA	AGAAAGAGAA	CTAAAAGACA
2101	CACAGGAAAA	TAAATCAAGAA	GATGACATTT	ACCAGTTAAT	TATGTAGTTT	AATAAAAAATA	AAAAATGGGA	CAAGTGAATA	TGGAGTCTTA	TCTGGTAGAC
2201	ACCTATCAAG	GCATCCCTTA	CACAGCAGT	GTTCAAGTTG	ATCTAGTAGA	AAAGGACCTG	TTACCTGCAA	GCCTAAACAAT	ATGGTTCCCC	CTGTTTCAGG
2301	CCAATACACC	ACCAGCAGTT	CTGCTTGATC	AGCTAAAAGAC	TCTGACTATA	ACTACTCTGT	ATGCTGCATC	ACAAAGTGGT	CCAATACTAA	AGTGAATGC
2401	ATCGGCCAG	GGTGCAGCAA	TGTCTGTACT	TCCCAAAAAG	TTTGAAGTCA	ATGGGACTGT	AGCATTTCAC	GAATATAGCA	AATTAGAAAT	TGACAAACTT
2501	ACAGTCTGTG	AAGTAAAAC	AGTTTACTTA	ACAACCATGA	AACCATATGG	GATGGTATCA	AAGTTTGTGA	GCCTGGCCAA	ACCAGTTGGC	AAAAAACAC
2601	ATGATCTAAT	CGCATTAATG	GATTTTATGG	ATCTAGAAA	GAACACACCA	GTTACAATAAC	CAGCATTTAT	CAATCAGTT	TCTATCAAGG	AGAGTGAATC
2701	AGCCACTGTT	GAAGCTGCAA	TAAAGCATGA	AGCAGACCAA	GCTCTAACAC	AAGCCAAAAT	TGCACCTTAT	GCGGACTGA	TCTATGATTA	GACCATGAAC
2801	AATCCCAAG	GCATATTCAA	GAAGCTTGGA	GCTGGGACCC	AAGTTATAGT	AGAACTAGGA	GCATATGTCC	AGGTGAAAG	CATAAGTAA	ATATGCAAGA
2901	TTGGAGGCA	TCAAGGAACA	AGATATGTGC	TGAAGTCCAG	ATAACAGCCA	AGCAACTGA	CCAAGAACTA	CCAACTCTAT	TCTATAGACT	AAAAAGTCG
3001	CATTTTAGTT	ATATAAAAAT	CAAGTTAGAA	TAAAGATTAA	ATCAATCAAG	AACGGGACAA	ATAAAAATGT	CTTGGAAAAT	GGTGATCAAT	TTTTTCATTC
3101	TAAATAACAC	TCAACACGGT	CTTAAAGAGA	GCTACCTTGA	AGATCATGT	AGCACTATAA	CTGAGGGATA	TCTTAGTGT	CTGAGGACAG	GTTGGTATAC
3201	CAACGTTTTT	ACATTAGAGG	TGGGTGATGT	AGAAAACCTT	ACATGTTCTG	ATGGACCTAG	CCTAATAAAA	ACAGAATTAG	ATCTGACCAA	AGTGCACATA
3301	AGAGAGCTCA	AAACAGTCTC	TGCTGACCAA	TTGGCAAGAG	AGGACAAAAT	TGAGATCCC	AGACAATCTA	GGTTTGTCT	AGGAGCAATA	GCACCTCGGTG
3401	TTGCAACAGC	AGCTGCAGTC	ACAGCAGGTG	TTGCAATTGC	CAAAACCATC	CGGCTTGTGA	GTGAAGTCAAC	AGCAATTAAG	AATGCCCTCA	AAACGACCAA
3501	TGAAGCAGTA	TCTACATTGG	GGAATGGAGT	TGGAGTGTG	GCAACTGCAG	TGAGAGAGCT	GAAAGACTTT	GTGAGCAAGA	ATTTAACTCG	TGCAATCAAC
3601	AAAAACAAGT	GCGACATTGA	TGACCTAAAA	ATGGCCGTTA	GCTTCAGTCA	ATTCACAGAG	AGGTTCTTAA	ATGTTGTGCG	GCAATTTTCA	GACAAATGCTG
3701	GAATAACACC	AGCAATATCT	TTGGACITAA	TGACAGATGC	TGAACCTAGCC	AGGCCGTTTT	CTAACATGCC	GACATCTGCA	GGACAAATAA	AATTGATGTT

Fig. 37A

HMPV strain 83 (continued)

3801 GGAGAACCGT GCGATGGTGC GAAGAAAGGG GTTCGGAATC CTGATAGGGG CTCTACGGGAG CTCCGTAAAT TACATGGTGC AGTGCCCAAT CTTTGGGGTT 3900
 3901 ATAGACACGC CTTGTCTGGAT AGTAAAGCA GCCCTTCTT GTTCCGGAAA AAAGGGAAC TATGCTTGCC TCTTAAAGAG AGACCAAGGG TGGTATTGTC 4000
 4001 AGAATGCAGG GTCAACTGTT TACTACCCAA ATGAGAAGA CTGTGAAAAC AGAGGAGACC ATGTCTTTTG CGACACAGCA GCGGGAATTA ATGTTGCTGA 4100
 4101 GCAATCAAAG GAGTGCACA TCAACATATC CACTACAAAT TACCCATGCA AAGTCAGCAC AGGAAGACAT CCTATCAGTA TGGTTGCAC GTCTCCTCTT 4200
 4201 GGGGCTCTGG TTGCTTGCTA CAAAGGAGTA AGCTGTGCCA TTGGCAGCAA CAGATAGGG ATCATCAAGC AGCTGAACAA GGGTTGCTCC TATATAACCA 4300
 4301 ACCAGATGC AGACACAGTG ACAAATAGCA ACATGTATTA TCAGCTAAGC AAGTTGAGG GTGACAGCA TGTATAAAA GGCAAGCCAG TGTCAAGCA 4400
 4401 CTTTGATCCA ATCAAGTTTC CTGAGATCA ATTCAATGTT GCATTGACC AAGTTTTTGA GAACATCGAA AACACAGG CCTTGGTAG TCAATCAAAC 4500
 4501 AGAATCTTAA GCAGTGCAGA GAAAGGGAAT ACTGGCTTCA TCATTGTAAAT AATTCTAAT GCTGTCTTG GCTCTAGAT GATCTAGTG AGCATCTCA 4600
 4601 TTATATATCAA GAAACCAAG AAACCAACGG GAGCACTCC AGAGCTGAGT GGTGTACAA ACAATGGCTT CATACCACAC AGTTPAGTTAA TTAAAAATAA 4700
 4701 AATAAAATTT GGGACAAATC ATATGTCTC GCAAGCTCC ATGCAAAAT GAACTGCGGG GCAATGCAA CAGAGGAAGT GAGTGAAGT TTAACCAAA 4800
 4801 TTACTGGAGT TGGCCAGATA GATACTTATT AATAAGATCA AACTATCTAT TAAATCAGCT TTTAAGGAAC ACTGATAGAG CTGATGGCCT ATCAATAATA 4900
 4901 TCAGGGCGAG GCAGAGAAGA CAGAACGCAA GATTTTGTTC TAGGTTCCAC CAATGTGGTT CAAGGTTATA TTGATGATA CCAAAGCATA ACAAAGCTG 5000
 5001 CAGCCTGCTA CAGTCTACAC AACATAATCA AGCAACTACA AGAAGTTGAA GTTAGGCAGG CTAGAGATAG CAAACTATCT GACAGCAAGC ATGTGGCCT 5100
 5101 CCATAACTTA ATCTTATCTT ACATGGAGT GAGCAAACT CCGCATCTT TAATCAACAA TCTCAAAAGA CTGCCGAGAG AAAAAGTGAA AAAATTAGCA 5200
 5201 AAGCTGATTA TTGACTTATC AGCAGGGGCT GACAATGACT CTTTATATGC CCTGCAAGC AGTGAAGCA TTAATCAAGT GCATGAGCA TGGTCCCTGTT 5300
 5301 TTCTATTACTA TAGAGTTTGA TGAATATGATA TGGACTCAA AAGAATTAAA AGAAGCTTTG TCCGATGGGA TAGTGAAGTC TCACACCAAC ATTTACAATT 5400
 5401 GTTATTTAGA AAACATAGAA ATTATATATG TCAAGGCTTA CTTAAGTTAG TAAAAACACA TCAGAGTGGG ATAAAGTGACA ATGATAACAT TAGATGTCAT 5500
 5501 TAAAAGTGAT GGGTCTTCAA AAACATGTAC TCACCTCAA AAAATAATCA AAGACCATTC TGGTAAAGTG CTTATTGAC CTTAAGTTAA ATTAGCTTTA 5600
 5601 CTAACATTTT TCACAATAAC AATCACTATA AATTACATAA AAGTAGAAAA CAATCTACAA ATATGCCAGT CAAAAACTGA ATCAGACAAA GAAGACTCAC 5700
 5701 CATCAAATAC CACATCCGTC ACAACCAAGA CCACTCTAGA CCATGATATA ACACAGTATT TTAATAAGATT AATTCAAAGG TATACAGATT CTGTAATAAA 5800
 5801 CAAGGACACA TGCTGGAAAA TAAGCAGAAA TCAATGCACA AATATAACAA CATATAAATT TTTATGCTT AAACCTGAG ACTCAAAAAT CAACAGTTGT 5900
 5901 GATCAGCTGA CAGATCTATG CAGAAACAAA TCAAAATCAG CAGCTGAAGC ATATCATACA GAGAAATGCC ATTCGATATA CACAATTGAG TGAAGAGTCT 6000
 6001 ATACCACTGC AATGATTATA ACCAATCTT AAGATTGAAA ACTAGACTAG GATCCGCTTA AGACTATCAG TTCAATAGTT TAGTTATTTT AAAATATTG 6100
 6101 AGAATAGGTA AGTTTCTATG GCATTCTATA GCAATAGGTA ATAAATTAACA GCTTAAATTAT AATTAAAAA TTTATTTAAA TCGTAACTAT TTAATTTACA 6200
 6201 AAGTAAAAAC AAAAATATGG GACAAGTAGT TATGGAGGTG AAAGTAGAGA ACATTGCGAG AATAGACATG CTCAAAAGCAA GAGTGAATAA TCGTGTGCA 6300
 6301 CGTAGCAAT GCTTTAAAAA TGCTTCTTTA ATCTCTATAG GAATAACTAC ACTGAGTATA GCTCTCAATA TCTATCTGAT CATAACTAC ACAATACAAA 6400
 6401 AAACCTCATC TGAATCAGAA CACCAACCA GCTCACACC CACAGAAATCC AACAGGAAG CTTCAACAAT CTCCACAGAC AACCCAGCA TCAATCCAAA 6500
 6501 CTCACAGCAT CCAACTCAAC AGTCCACAGA AAACCCACCA CTCAACCCCG CAGCATCAGT GAGCCCATCA GAAACAGAAC CAGCATCAAC ACCAGACACA 6600
 6601 ACAAACCGCC TGTCTCTCCGT AGACAGGTCC ACAGCACAA CAAAGTGAAG CAGAACAAAG ACAAAACCGA CAGTCCACAC AAGAAACAA CCAAGCACAG 6700
 6701 CTTCCAGTAC ACAATCCCA CCACGGCAA CAACGAGGC AATCCGAGA GCCACCACTT TCCGCTATG CAGCACAGAG AAAAGACCAA CCACAACATC 6800
 6801 AGTCCAGTCC GACAGCAGCA CCACAACCCA AAATCATGAA GAACACAGTT CAGGAAACC ACAGGATCT GTAAGCAGAA TGCAAAAC TA GCACACCAAC 6900
 6901 AATATAAACC CAAATAGITT AACAAAAAT ACAGATAGC TCTAAAGTAA AACATGTAG TACCACAAT CAAGAAACCA AAGACAACT CACAATCTCC 7000
 7001 CTAAAAACAGC AACGACACCA TGTCACTTT GCTCAATCT CTCTGGGAGA AACTTTTGCC CACATACTAA CAACATCACA ACCATCTCA ACCATCTCA 7100
 7101 TGGGCAAAAC AGATCTCAAG AGACAAATAG CAATGGATCC TCTTAATGAA TCCACTGTTA ATGCTATCT CCTGATTCG TACCTTAAAG GAGTAATTC 7200
 7201 TTTTAGTCAA ACTTAATGCAA TTGTTTCAAT TCTCTTAAA AGACCTTACT TAAAAAATGA CAACACTGCA AAGTTGCCA TAGAATCC TAGAATCC 7300
 7301 CATGTAGAC TCAAAAATGC AGTCAATCT AAAATGAAA TATCAGATTA CAAGGTAGTA GAGCCAGTAA ACATGCAACA TGAATAATG AAGAATGTAC 7400
 7401 ACAGTTGTGA GCTCACACTA TTGAAACAGT TTTTAAACAG GAGTAAAAAC ATTAGCACTC TCAAAATTGAA TATGATATGT GATTGGCTGC AATTAAAGTC 7500
 7501 TACATCAGAT GATACCTCAA TCCTAAGTTT CATAGATGTA GAATTTATAC CTAGTTGGGT AAGCAACTGG TTTAGTAAAT GGTACAATCT CAATAAGTAA 7600
 7601 ATTTTGAAT TCAGAAGAGA GGAAGTAATA AGAACCGGTT CAATCTTATG CAGGTCATTG GGTAAATTAG TTTTATTGTT ATCATCATAT GGATGTATCG 7700
 7701 TCAAGACAA CAAAAGCAA AGAGTAGCT TCTTCACATA CAATCAACTG TTAACATGGA AAGATGTGAT GTTAAAGTAG TTTAATGCGA ATTTTGTGAT 7800

Fig. 37B

HMPV strain 83 (continued)

7801	ATGGGTAAGC	AATAGTCTGA	ATGAAAAATCA	GGAAGGGCTA	GGGTGAGAA	GTAATCTGCA	AGGTATGTTA	ACTAATAAAC	TATATGAAAC	TGTAGATTAT	7900
7901	ATGCTAAGCT	TATGTTGCAA	TGAAGGTTTC	TCACCTTGGA	AGAGTTTCGA	AGGTTTATTT	ATGAGTACAG	TCCTTAGGAT	TACTGAACAT	GCTCAATTCA	8000
8001	TAATAGATT	TAGAAAATACT	TTATTGAATG	GATTAAACAT	TCAATTAAAC	AAATTAACAA	ATAAAACAGC	ACTCAGAGTT	CATAGTACCG	TATTAGAAAA	8100
8101	TAATGATTAT	CCAATGTATG	AAGTTGTACT	TAAATATATA	GGAGATACTT	TGAGATGTTAT	CAAAATTATTA	ATCAATAAAA	ACTTAGAGAA	TGCTGCAGAA	8200
8201	TTATATCTATA	TATTCAGAAAT	TTTTTGGTCAAT	CCAAATGTFAG	ATGAAAGAGA	TGCAATGGAT	GCTGTCAAAT	TAAACAATGA	AATCAGAAA	ATCCTAAGGT	8300
8301	TGGAGAGCTT	GACAGAACTA	AGAGGGCAT	TCATATTAAAG	GAATATCAAA	GGATTGTG	ACAACACAA	AGGTTGGCC	AAATTAATAA	ACTTAAAGT	8400
8401	GCTTAGCAAA	AGATGGACTA	TGTACTTCAA	AGCTAAATAAT	TACCCAGTC	AACCTGAAAT	AAGTGAACAA	GACTTTCTAG	AGCTTGTCTG	AATACAATTT	8500
8501	GAACAAGAGT	TTTTCTGTCC	TGAATAAAC	AACTCTGAGA	TGGTATTAAA	TGACAAAGCC	ATATCACCCT	CTAAAGAGATT	AATATGGTCT	GTGTATCCAA	8600
8601	AGAACTACTT	ACTTGAGACG	ATAAAAATC	GATATTTAGA	AGAACTTTTC	AATGCGAGTG	ATAGTCTCAA	AAACAAGAAG	GTACTAGAGT	ACTATTAAAA	8700
8701	AGATAATAAA	TTTTGATCAA	AGGAACCTAA	AAGTTATGTA	GTTAGACAAG	AAATATTAAA	CGATAAGGAG	CACATTTGCT	CATTAACTGG	AAAAGAAGA	8800
8801	GAATTAAGTG	TGGTAGAAT	GTTTGCTATG	CAACCAGGAA	AACAGCGACA	AATACAAATA	TTGGCAGAAA	AAATGTTAGC	TGATAACATT	GTACCTTTCT	8900
8901	TCCCGGAAC	CTTAACAAAG	TATGGTGATC	TAGATCTTCA	GAGAAATATG	GAATCAAAAT	CAGAACTTTT	TTCTATCAAA	ACCAGAGAA	ATGATAGTTA	9000
9001	TAATAATTAC	ATTGCAAGAG	CATCCATAGT	AACAGATTTG	AGCAAGTTCA	ACCAAGCCTT	TAGATATGAA	ACTTAGCCGA	TCTGTGCGGA	TGTAGCAGAG	9100
9101	GAATTACATG	GAACACAAAG	CTTATTCTGT	TGGTTACATC	TTATCGTTCC	TATGACTACA	ATGATATGTG	CTTATAGACA	TGCACCACCA	GAACACAAAG	9200
9201	GTGAATATGA	TATAGATAAG	ATAGAAAGAGC	AAAGTGGTCT	ATATAGATAT	CACATGGGCG	GTATTGAAGG	ATGGTGTCAA	AACTCTGGA	CAATGGAAGC	9300
9301	TATATCTTTA	TTGGATGTTG	TATCTGTAAA	GACACGGTGT	CAATGACAT	CTTTTATAAA	CGGTGACAA	CAATCAATAG	ATGTAAGTAA	ACCAGTCAAG	9400
9401	TTATCTGAAG	GTTTAGATGA	AGTGAAGCA	GATTATCGT	TAGCAGTAAA	AATGCTAAAA	GAATAAAGAG	ATGCATACAG	AAATATAGGC	CATAAECTTA	9500
9501	AAGAAGGGGA	AACATATATA	TCAAGGGATC	TTCAGTTTAT	AAGCAAGGTG	ATTCAATCTG	AAGGAGTGAT	GCATCTTACC	CCTATAAAAA	AGGCTTTGAG	9600
9601	AGTAGGACCA	TGGATAAACA	CAATATTAGA	TGACATTAAA	ACTTATGCTG	AGTCAATAGG	GAGTCTATGT	TAGGAATTTAG	AAATTAGGGG	AGAAAGCATTA	9700
9701	ATAGTTACTG	TGATATTAA	AACTTCTGG	CTGTATAACT	TATACATGCA	TGAATCAAA	CAACATCTTT	CGAGAGGGAA	ACAGTATTTC	AAACAACATA	9800
9801	ATAAAACATT	AACATCAGTG	CAGAGATTTT	TTGAAAATTAA	AGGGAAAAAT	GAGGTAGTAG	ATCTATGGAT	GAACATACCA	ATGCAATTTG	GAGGAGGAGA	9900
9901	TCAGTAGTTC	TTCTATAGAT	CTTTCTATAG	AAGGACCCCT	GATTTTTTAA	CTGAGGCAAT	CAGCCATGTA	GATATTCTGT	TAAAAATATC	AGCTAACATA	10000
10001	AAAAATGAAA	CGAAAGTAA	TTTTCTTCAA	GCTTACTAT	CAATFAGAAA	AAATGAACGT	GCTACACTGA	CAACACTAAT	GAGAGATCCT	CAAGCTGTGT	10100
10101	GATCAGAACG	ACAGCAAAA	GTAACAAGTG	ACATCAATAG	AACAGCAGTT	ACCAGTATCT	TAACTCTTTT	CCCAAAATCAA	CTTTTCAGTG	ATAGTGTCTAT	10200
10201	ACACTACAGC	AGAAATGAAG	AAGAAGTGGG	AATCATTTGCA	GAACACATAA	CACCTGTTTA	TCCTCATGGG	CTGAGAGTAT	TATATGAATC	ATTGCCCTTT	10300
10301	CACAAAGCTG	AAAAAGTTGT	AAACATGATA	TCAGGGGACAA	AATCTATAAC	CAACTTATTA	CAGAGAACAT	CCGCTATTAA	TGGTGAAGAT	ATTGACAGGG	10400
10401	CTGTATCTAT	GATFTTGGAG	AATCTTAGAT	TATATCTAG	AAATTTGTCA	GTAGTTGTTG	ATAGTATAGA	AAATCCAATC	AAATCTAATG	GTAGGCTGAT	10500
10501	ATGTTGTCAA	ATCTCTAGGA	CTTTAAGAGA	GACATCATGG	AAATAATATG	AAATAGTTGG	AGTAACATCT	CCTAGCATCA	CTACATGTAT	GGATGTCTAT	10600
10601	TATGCAACTA	GTTCTCATTT	GAAAGGATA	ATTATAGAAA	AGTTACAGC	TGACAGAACT	ACAAGGGTTC	AAAGAGTCC	AAAAAGCCTT	TGGGTAGGTT	10700
10701	CGAGTACTCA	AGAGAAAAAA	TTAGTACTTG	TTTATAACAG	ACAAATTTCT	TCAAAAACAAC	AAAGAGAAAC	GCTAGAAGCA	ATTGAAAAAA	TGAGATGGTT	10800
10801	GTATAAAGGG	ACACCAGGCT	TGCGACGAT	ACTCAACAG	ATCTGTCTTG	GGAGTTTAGG	CATTAGCTAC	AAATGTGTAA	AACCTTTATT	ACCTAGGTTT	10900
10901	ATGAGTGTAA	ATTCTTTACA	TAGATTATCT	GTCAGTAGTA	GACCTATGGA	ATTCACAGCA	TCAGTTCCAG	CTTATFAGAAC	AACAAATFAC	CATTTCCGACA	11000
11001	CTAGTCCCTAT	TAATCAAGCA	CTAAGTGAGA	GATTTGGGAA	TGAAGATATT	AACCTGGTCT	TCCAAAATGC	AATCAGCTGT	GGAAATTAGCA	TAAATGAGTT	11100
11101	AGTAGAACAA	TTACACAGGA	GAAGCCCAA	ACAGTTAGTT	TTAATACCCC	AAATTAGAAGA	AATAGACATT	ATGCCACCAC	CAGTGTITCA	AGGGAATTC	11200
11201	AATATATAAT	TAGTAGATAA	GATAACTTCT	GATCAACATA	TCCTTAGTCC	GGACAAAATA	GATATTGTAA	CACFAGGGAA	AATGTCATG	CCCACTATAA	11300
11301	AAGGTCAGAA	AACAGATCAG	TTCTTAAATA	AGAGAGAAAA	TTATTTCAT	GGAAACAATC	TTATTGAGTC	TTTATFAGCA	GCATTAGCAT	GTATGTGGTG	11400
11401	TGGGATATTA	ACAGAACAA	GCATAGAAA	TAAATATTTT	AAGAAGACT	GGGTCAGCGG	GTATTATCA	TATCATGCTT	TTATGGACTT	CAAAATATTC	11500
11501	CTATGTGCTT	TTAAAACTAA	ACTTTATAGT	AGTTGGGGG	CTCAAGGGAA	AAACATATAA	GATGAAGATA	TAGTAGATGA	ATCAATAGAT	AAATTTGTTAA	11600
11601	GGATGTACAA	TACTTTTGG	AGAATGTCA	GCAAGGTTAT	GTTTGAACCA	AAGGTTAAGA	AAAGGATTAAT	GTTTATATGAT	GTAAAAATCC	TATCACTAGT	11700
11701	AGGCTACATA	GGGTTTAAAG	ACTGGTTTAT	AGAGCAGTTG	AGATCAGCTG	AAATTGCATGA	AATACCTTGG	ATTGTCAATG	CCGAAGGTGA	TTTGGTTGAG	11800

Fig. 37C

HMPV strain 83 (continued)

11801	ATCAAGTC	TTAAATCTA	TTTGCAACTG	ATAGAACAAA	GCCTTATTTT	AAGAATAACT	GTTTTGAAC	ATACAGATAT	GGCACATGCT	CTCACACGAT	11900
11901	TAAATCAG	GAAGTTAATG	TGTGATAATG	CACTGTTTAC	CCCAATTTCA	TCCCCAATGG	TTAACTTTAC	TCAAGTTAT	GATCCACAA	CACAATTAGA	12000
12001	TTAATTTCCC	AAGATAACAT	TCGAAAGGCT	AAAAAATTAT	GACACAAGTT	CAAAATTATGC	TAAAGGGAAG	CTAACCAAGAA	ATTACATGAT	ACTATTGCCA	12100
12101	TGGCAGCATG	TTAATAGATA	TAACTTTGTC	TTTAGTTCTA	CTGGATGTAA	AGTTAGTCTG	AAAACATGTA	TTGGAAAACT	TATGAAAGAC	CTAAATCCTA	12200
12201	AAGTTTGTGA	CTTTATTGGA	GAAGGAGCAG	GAAATTGGAT	GGCCAGAAACA	GCATGTGAAT	ATCCTGATAT	TAAATTTGTA	TATAGAGTC	TGAAAGATGA	12300
12301	CCTTGATCAT	CATTATCCTC	TGGAATACCA	GAGAGTGATA	GGTGAATTAA	GCAGAAATCAT	AGATAGTGGT	GAAGACTTTT	CAATGGAAAC	AACAGACGCA	12400
12401	ACTCAAAAAA	CTCATTTGGG	TTTGATACAC	AGGGTAAGCA	AAGATGCTTT	ATTAATAAAT	TTATGTGATG	CAGAAATTTAA	GGACAGAGAT	GATTTTTTTA	12500
12501	AGATGGTAA	TCTATGGAGA	AAACATGTAT	TATCATGCAG	AATTGCACT	ACTTATGGGA	CGGACCTCTA	TTTATTCGCA	AAGTATCATG	CTAAAGACTG	12600
12601	CAATGTAAAA	TTACTTTTTT	TTGTGAGATC	AGTTGCTACT	TTTCATTATGC	AGGGTAGTAA	GCTGTCAAGT	TCAGAAATGCT	ACATACTCTT	AACACTAGGC	12700
12701	CACCACAA	GTTTACCTTG	CCATGGAGAA	ATACAAAAAT	CTAAGATGAA	AATAGCAGTG	TGTAATGAT	TTTATGCTGC	AAAAAACTC	GACAATAAAT	12800
12801	CAATTGAGC	TAAATGTAAA	TCACTTTGT	CAGGGCTAAG	AAATACCTATA	AAATAAGAGG	AACATAGTAG	ACAGAGAAGA	TTATTAAAC	TACAAAGCAA	12900
12901	TCATTCTTCT	GTAGCAACAG	TTGGCGGTAG	CAAGATCATA	GAGTCTAAGT	GGTTAACAAA	CAAAAGCAAGT	ACAATAATTG	ATTGGTTAGA	ACATATTTTA	13000
13001	AATCTCCAA	AGGGGAATT	AAATTATGAT	TTTTTTTGAAG	CATTGGAGAA	CACCTTACCCT	AAATGTGATTA	AACTAATAGA	TAACTTAGGG	AATGCAGAGA	13100
13101	TTAAAAAACT	GATCAAAAGTA	ACAGGATACA	TGCTTGTGAAG	TAAAAAATGA	AAAATGATGA	AGATGACAAA	ATAGATGACA	ACTTCATACT	ATTCTAAAT	13200
13201	AATTATTTGA	TTATGCAATT	ATATGATAGT	TAATTAAAAAT	TAAAAAATTAA	AAATCAAAAG	TTAAAAITTTA	AAACCTATCA	TTAAGTTTAT	TAAAAATAAG	13300
13301	AAATTATAAT	TGAATGTATA	CGGTTTTTTTT	GCCGT							13335

Fig. 37D

rHMPV-GFP

1 ACACGAAAAA AACCAGTATA AATTAACTTA CAAAAAACA TGGGACAAGT 50
 101 TGGTCGAGCT GGACGGCGAC GTAAACGGCC ACAAGTTTCA CGTGTCGGG 100
 201 GACTACCGGC AAGCTGCCCG TGCCCTGGCC CACCTCGTG ACCACCTTGA 200
 301 GACTTCTTCA AGTCGCGCCAT GCCCGAAGGC TACGTCGAG AGCGACCAT 300
 401 AGGGCGACAC CCTGTGTAA CCGATCGAAC TGAAGGGCAT CGATTCAAG 400
 501 CAACGTCTAT ATCATGGCCG ACAAGCGAA CAAAGGGCAT AAGTTGAAT 500
 601 TACACGCTAT ACACGCCAT CGGCGACGC CCCTGTCTG TGCCTGCA 600
 701 GCGATCAAT GGTCTGTCTG GAGTCTGTGA CACCTGAGTG ATCTATCA 700
 801 AAATGTCTCT TCAGGGGATT CCCTCATCAT TGCAACAAGA AATAACACT 800
 901 AGCAGTGACA CCCTCATCAT TTAGCACAGC TCTAGGATCA GAGAGAGTAC 900
 1001 ATACAAATATA TTAGCACAGC TCTAGGATCA GAGAGAGTAC AGCAGATTCT 1000
 1101 TGGGGAAGT TAAACCAAC AAAGGAGAAG ATTACAGAT GTTTAGACATA 1100
 1201 AACAAATGGA CTTTGTCTTA AAGAAATCAT AGGCAATATT CCACAAAATC 1200
 1301 TTAATAATTTA CCAAACTAGC ATCAACTATA GAAGTGGGAT TAGAGACCAC 1300
 1401 GGATGGACAT ACCAAAAATC GCTAGATCTT TCTATGATTT ATTGAAACA 1400
 1501 ATCTCTTACA GGCAGCAAG CAGAAAGTTT ATTCTGTTAT ATATTCAATG 1500
 1601 TCATCTAACA ATATAATGTT AGGACATGTA TCTGTCCAAG CTGAGTTAAA 1600
 1701 GGTCTCTTACA TTTTAAAGGCAA AGCCCAAAAG CTGGACTGTT ATCACTAGCC 1700
 1801 CATAATAGAT ATGTATCGCG GGAGAGTGCC AAACACAGAA CTAATTTTCAG 1800
 1901 TCTTCAATAG GACTCACAGA TGAAGAAAAA GAGGTGCGAG AACACTTTCT 1900
 2001 ACAATCAAAA ATGTCATTC ATCTCAATC TATTATAGGA GAAAAAGTGA 2000
 2101 AGTCATCAAAA ATGTCATTC ATCTCAATC TATTATAGGA GAAAAAGTGA 2100
 2201 TGTCAGAGCC GAAGTTAGCA TGGACACAGA CAGGTGCGAG AATCAAAAAT 2200
 2301 TACTGAGAAA AGGTGTCTGC CCTCCAGTGA AAGTTGGAG AAAGATGCTC 2300
 2401 AATGAACACAG GAAATACAC AAAGTTGAG AGCATTTGAAG CCAGACTAGA 2400
 2501 AAAGAGATAC TTTTATCATTA AGCATTTGAAG AGCATTTGAAG CCAGACTAGA 2500
 2601 AGCAGGACCC ACAGCAGCAA GAGATGGGAT CAGAGATGCA ATGATTTGGCA 2600
 2701 GCAGAAATGA TGGAAAGAAGA AATGAACCCAG CCGACAAAAA TAGGAAAACGG 2700
 2801 ACAGAGACAC AAGTGTGAA TCCGAGAAG AGTGAATAAG GAGTCTTATC 2800
 2901 TAAATAAATA AATGTTGAGC AGTGAATAAG GAGTCTTATC TGGTAGACAC 2900
 3001 AGGACCTGTT ACCTGCAAGC CTAACAATAT GGTTCCTCTT GTTTCAGGCC 3000
 3101 TACTCTGTAT GCTGCATCAC AAAGTGTCTC AATACTAAAA GTGAATGCAT 3100
 3201 GCGACTGTAG CACTTGACGA ATATAGCAAA TTAGAATTG ACAACTTAC 3200
 3301 TGGTATCAAA GTTTGTGAGC TCGGCCAAAC CAGTTGGGAA AAAACACAT 3300
 3401 TACAATACCA GCATTATCA AATCAGTTTC TATCAAGAG AGTGAATCAG 3400
 3501 GCCAAATATG CACTTATGC GGGACTGATC ATGATTTATGA CCATGAACAA 3500
 3601 AACTAGAGC ATATGTCCAG GCTGAAAGCA TAAAGTAAAT ATGCAAGACT 3600
 3701 CAACCTGACC AAGAATACC AACTCTATTC TATAGACTAA AAAGTCGCCA 3700

Fig. 38A

rHMPV-GFP (continued)

3801	CGGACAAAT	AAAAATGTCT	TGGAAAGTGG	TGATCATTTT	TTCAATTGCTA	ATAACACCTC	AACACGGTCT	TAAAGAGAGC	TACCTAGAAG	AATCATGTAG	3900
3901	CACTATAACT	GAGGATATC	TTAGTGTTC	GAGGACAGGT	TGGTATPACCA	ACGTTTTTAC	AATTAGAGGTG	GGTGATGTAG	AAAACCTTAC	ATGTTTCTGAT	4000
4001	GGACCTAGCC	TAATAAAAAC	AGAAATTAGT	CTGACCAAAA	GTGCACTAAG	GAGACTCAAA	ACAGTCTCTG	CTGACCAATT	GGCAAGAGAG	GAACAAATTG	4100
4101	AGAAATCCCG	ACAATCTAGG	TTTGTTCCTAG	GAGCAATAGC	ACTCGGTGTT	AGAACAGCAG	TCGCAGTCTAC	AGCAGGTGTT	GCAATTGGCA	AAACCATCCG	4200
4201	GCTTGAGAGT	GAAGTCACAG	CAATTAAGAA	TGCCCCTCAA	ACGACCAATG	AAGCAGTATC	TACATTGGGG	AATGAGTTTC	GAGTGTGGC	AACCTGCATG	4300
4301	AGAGAGCTGA	AAGACTTTGT	GAGCAAGAA	TTAACTCGTG	CAATCAACAA	AAACAAGTGC	GACATTGGG	ACCTAAAAAT	GGCGTTAGC	TTTCACTCAAT	4400
4401	TCAACAGAA	GTTTCTAAAT	GTGTGCGGC	AATTTTCAGA	CAATGCTGGA	ATAACACACG	CAATATCTTT	GGACTTAATG	ACAGATCTG	AATAGCCAG	4500
4501	GGCGGTTTCT	AACATGCCGA	CATCTGCAGG	ACAAATAAAA	TTGATGTTGG	AGAACCCGTG	GAPGTGCGA	AGAAAGGGT	TCGGAATCCT	GATAGGGGTC	4600
4601	TACGGGAGCT	CCGTAATTTA	CATGTGTCAG	CTGCCAATCT	TTGGCGTTAT	AGACACGCT	TGCTGGATAG	TAAAGCAGC	CCCTCTTGT	TCGGAAAAA	4700
4701	AGGAAACTA	TGCTTGCCTC	TTAAGAGAAG	ACCAAGGGTG	GTATTTGTGAG	AATGCAGGGT	CAACTGTTTA	CTACCCAAAT	GAGAAAGACT	GTGAAACAAG	4800
4801	AGGAGACCAT	GTCTTTTGGG	ACACAGCAGC	GGGAATTAAT	GTTCCTGAGC	AATCAAGGA	GTGCAACATC	AACATATCCA	CTACAAATTA	CCCATGCAAA	4900
4901	GTCAGCACAG	GAAGACATCC	TATCAGTATG	TTTGCACTGT	CTCTCTTTGG	GGCTCTGGTT	GTCTGCTACA	AAGGAGTAAG	CTGTTCCATT	GGCAGCAACA	5000
5001	GAGTAGGGAT	CATCAAGCAG	CTGAACAAGG	GTTCCTCTTA	TATAACCAAC	CAAGATGCAG	ACACAGTGC	AATAGACAAC	ACTGTATATC	AGCTAAGCAA	5100
5101	AGTTGAGGGT	GAACAGCATG	TTATAAAGG	GTTGCTCCTA	TCAAGCAGCT	TTGATCCAA	CAAGTTTCTT	GAAGATCAAT	TCAATGTTC	ACTTGACCAA	5200
5201	GTTTGTGAGA	ACATTGAAAA	CAGCCAGGCC	TTGGTAGATC	AATCAAAACAG	AATCTTAAGC	AGTGCAGAGA	AAGGGAATAC	TGGCTTTCATC	ATTGTAATAA	5300
5301	TTCTAAATTGC	TGTCCTTGGC	TCTAGCATGA	TCCTAGTGAG	CATCTTCATT	ATAATCAAGA	AAACAAAGAA	ACCAACGGGA	GCACCTCCAG	AGCTAGTGG	5400
5401	TGTCACAAAC	AATGGCTTCA	TACCACACAG	TTAGTTAAAT	AAAAATAAAA	TAAATTTTGG	GACAAATCAT	AATGCTCTCG	AAGGCTCCAT	GCAATATAGA	5500
5501	AGTCGGGGC	AAATGCAACA	GAGGAAGTGA	GTGTAAGTTT	AACCACAATT	ACTGGAGTTG	GCCAGATAGA	TACTTATTA	TAAGATCAAA	CTATCTATTA	5600
5601	AATCGCTTTT	TAAGGAACAC	TGATAGAGCT	GATGGCCTAT	CAATPAATATC	AGGCGCAGGC	AGAGAAGACA	GAACGCAAGA	TTTTTGTCTA	GGTTCACCA	5700
5701	ATGTGCTTCA	AGGTTATATT	GATGATAACC	AAAGCAATAC	AAAAGCTGCA	GCCTGCTACA	GTCTACACAA	CATAATCAAG	CAACTACAAG	AAGTTGAAT	5800
5801	TAGGCAGGCT	AGAGATAGCA	AACATCTGA	CAGCAAGCAT	GTGGACTCC	ATACTGTAAT	CTTATCTTAC	ATGAGATGA	GCAAACTCC	CGCATCTTTA	5900
5901	ATCAACAAATC	TCAAAAGACT	GCCGAGAGAA	AAACTGAAA	AATTAGCAAA	GCTGATAATT	GAGTTTGATG	CAGGCGTGA	CAATGACTCT	TCATATGCC	6000
6001	TGCAAGACAG	TGAAGCAATT	AATCAAGTGC	AGTGAGCATG	GTCTGTTTTT	CATTACTATA	GAGTTTGATG	AATGATATG	GACTCAAAA	GAATTAAAG	6100
6101	AAGCTTTTTC	CGATGGGATA	GTGAAGTCTC	ACACCAACAT	TTACAATTGT	TATTTAGAAA	ACATAGAAAT	TATATATGTC	AAGGCTTACT	TAAGTTAGTA	6200
6201	AAAACACATC	AGAGTGGGAT	AAGTGACAAT	GATAACATTA	GATGTCATTA	AAAGTATGG	GTCTTCAAAA	ACATGTACTC	ACCTCAAAA	AATAATCAAA	6300
6301	GACCATTTCTG	GTAAAAGTGCT	TATTGCACCTT	AAGTTAATAT	TAGCTTTACT	AACATTTTTC	ACAATAACAA	TCATATAAA	TTACATAAAA	GTAGAAAAA	6400
6401	ATCTACAAAT	ATGCCAGTCA	AAAACCTGAAT	CAGACACAAGA	AGACTCACCA	TCAAATPACCA	CATCCGTCAC	AACCAAGACC	ACTTAGACC	ATGATATTAAC	6500
6501	ACAGTATTTT	AAAGATTAA	ACCTGAGGAC	TACAGATTCT	GTAAATAACA	AGGACACATG	CTGGAAAAATA	AGCAGAAATC	AATGCACAAA	TATAACAACA	6600
6601	TATAAATTTT	TATGCTTTAA	ACCTGAGGAC	TCAAAAATCA	ACAGTTGTGA	TAGACTGACA	GATCTATGCA	GAACCAAAATC	AAAATCAGCA	GCTGAAGCAT	6700
6701	ATCATACAGT	AGAAATGCCAT	TGCATATACA	CAATTGAGTG	GAAGTGTAT	CACCACTCAA	TAGATTAAAC	CCAATCTTGA	ATGTTAAAAAC	TAGACTAGGA	6800
6801	TCCGTCTAAG	ACTATCAGTT	CAATAGTTTA	GTATTTTAA	AATATTTGAG	AATAGGTAAG	TTTCTATGGC	ACTTCATAGC	AATAGGTAAT	AATTAACAGC	6900
6901	TTAATTATAA	TTAAAACAATT	ATTTAAAATC	GTAACTATTT	AAATTACAAA	GTAAAAAACAA	AAATATGGGA	CAAGTAGTTA	TGGAGGTGAA	AGTAGAGAAC	7000
7001	ATTCGAGCAA	TAGACATGCT	CAAAGCAAGA	GTGAAAATC	GTGTGGCAG	TAGCAAAATGC	TTTAAAAATG	CTTCTTTAAT	CCTCATAGGA	ATAACTACAC	7100
7101	TGAGTATPAG	TCTCAATATC	TATCTGATCA	TAAACTACAC	AATACAAAAA	ACCTCATCTG	AATCAGAAC	CCACACAGC	TCACACCCCA	CAGAATCCAA	7200
7201	CAAGGAGCT	TCAACAATCT	CCACAGACAA	CCGAGACATC	AATCCAACT	CACAGCATCC	AACTCAACAG	TCACAGAAA	ACCCACACT	CAACCCCGCA	7300
7301	GCATCATGTA	GCCCATCAGA	ACAGAACCA	GCATCAACAC	AATCCAACT	AAACCGCTG	TCCTCCCTAG	ACAGTCCAC	AGCAACACCA	AGTGAAGCA	7400
7401	GAACAAAGAC	AAAACCGACA	GTCCAACAA	GAACACACC	AAGCAGACT	TCCAGTACAC	AATCCCCACC	ACGGGCAACA	ACGAAGGCAA	TCCGACAGC	7500
7501	CACCACTTTC	CGCATGAGCA	GCACAGGAAA	AAGACCAACC	ACACATCTAG	TCAGTCCGA	CAGCAGCACC	ACAACCCAAA	ATCATGAAGA	AACAGGTTCA	7600
7601	GCGAACCCAC	AGGCATCTGT	AAGCACAATG	CAAACTTAGC	ACACCAACAA	TATAAAACCA	AATTAGTTAA	CAAAAATATAC	GAGATAGCTC	TAAAGTAAAA	7700
7701	CATGTAGGTA	CCAACAATCA	AGAAACCAAA	AGACAACTCA	CAATCTCCCT	AAAACAGCAA	CGACACCATG	TCAGCTTTGC	TCAATCTCT	CTGGGAGAAA	7800

Fig. 38B

rHMPV-GFP (continued)

7801	CTTTTGGCCCA	CATACCTAAC	ACATCACCAAC	CATCTCAAGA	AAAGAAACTG	GGCAAAACAG	CATCCAAGAG	ACAAATAGCA	ATGGATCCTC	TTAATGAATC	7900
7901	CACCTGTTAAT	GTCTATCTCC	CTGATTCGTA	CCTTAAAGGA	GTAATTTCTT	TTAGTGAAC	TAATGCAAT	GGTTCAATGC	TCTTAAAAAG	ACCTACTTTA	8000
8001	AAAAATGACA	ACATGCGAAA	AGTTGCCATA	GAGATCTCTG	TTATTGAGCA	TGTTGAGACT	AAAAATGCAG	TCAATTTCTAA	AATGAAATA	TCAGATTACA	8100
8101	AGTTAGTAGA	GCCAGTAAAC	ATGCAACATG	AAATAATGAA	GAATGTACAC	AGTTGTGAGC	TCACACTAAT	GAACAGATTT	TTAACACAGG	GTAACAAATC	8200
8201	TAGCACTCTC	AAATTGAATA	TGATAATGTA	TTGGCTGCAA	TTAAAGTCTA	CATCAGATGA	TACCTCAATC	CTAAGTTTCA	TAGATGTAGA	ATTATATACCT	8300
8301	AGTTGGGTAA	GCAACTGGTT	TAGTAATTTG	TACAATCTCA	ATAAGTTAAT	TTTGGAAATC	AGNAGAGAGG	AGTAAATAAG	AACCGGTCA	ATCTTATGCA	8400
8401	GGTCATTGGG	TAAATAGTT	TTTATTGTAT	CATCATATGG	ATGTATCGTC	AAAGACCAAC	AAAGCAAAAG	AGTGAGCTTC	TTACATACA	ATCAACTGTT	8500
8501	AAATCTGCAAG	GATGTGATGT	TAAATAGATT	TAATGCGAAT	TTTTGTATAT	GGGTAAAGCA	TAGTCTGAT	GAATACTCAG	AAGGGCTAGG	GTTGAGAAGT	8600
8601	ATTTTATTAT	GAGTGAGATC	TAATAAACTA	TATGAACTG	TCAATTCAGT	GCTAAGCTTA	TGTTGCAATG	AAGTTTCTC	ACTTGTGAAA	GAGTTCGAAG	8700
8701	ATTTAAAAAT	AAAACACAGC	TCAGAGTTCA	TAGTACCGTA	TTAGAAAATA	ACTAGATTTC	GAATACTTTT	ATTGAATGGA	TTAACAGATC	AATTAACAAA	8800
8801	AGATGTATCA	AATTTATTAAT	CAATAAAAC	TTAGAGAATG	CTGCAAAAT	ATACTATATA	TTCAGAAATTT	TTGGTCATCC	AATGTAGAT	GAAGAGATG	8900
9001	CAATGTGATC	TGTCAAATTA	AACAATGAAA	TCACAAAAAT	CCTAAGGTTG	GAGAGCTTGA	CAGAACTAAG	AGGGCATTC	ATATTAGGA	TTATCAAAAG	9100
9101	ATTTGTGGAC	AACAACAAA	GGTGGCCAA	TTAAATAAAC	TTAAAGTGC	TTAGCAAAAG	ATGGACTATG	TACTTCAAAG	CTAAAATA	CCCAGTCAA	9200
9201	CTCGAATTTA	GTGAACAAGA	CTTCTAGAG	CTTGCTGCAA	TACAAATTGA	ACAAGAGTTT	TCTGTCTCTG	AAAAAACCAA	TCTTGAGATG	GTATTAAATG	9300
9301	ACAAAGCCAT	ATCACCTCCT	AAAAGATTAA	TATGCTCTGT	GTATCCAAAG	AATTACTTAC	CTGAGACGAT	AAAAAATCGA	TATTTAGAAG	AAACTTTCAA	9400
9401	TGCGAGTGAT	AGTCTCAAAA	CAAGAAGAGT	ACTAGAGTAC	TATTTAAAG	ATAATAAAT	TGATCAAAG	GAACCTAAAA	GTATTGTAGT	TAGACAAGAA	9500
9501	TATTTAAACG	ATAAGGAGCA	CATTGTCTCA	TTAACTGGAA	AAGAAAGAGA	ATTAAGTGTA	GGTAGAATGT	TTGTATGCA	ACCAGAAAA	CAGCGACAAA	9600
9601	BACAAATATT	GGCAAAAAA	TTGTTAGCTG	ATPACAAATGT	ACCTTTCTTC	CCGAAAACT	TAAACAAAGTA	TGGTGATCTA	GATCTTCAGA	GAATAATGGA	9700
9701	TAACCAATTA	GAATTTCTTT	CTATCAAAC	CAGAGAATAAT	GATAGTTATA	ATAATACAT	TGCAAAAGT	TATTTCTGTTG	GTTCATCTTT	ATCGTTCCCTA	9800
9801	CAAGCCCTTTA	GATATGAAC	TACAGCGATC	TGTGCGGATG	TAGCAGACGA	GAATATGATA	TAGATAAGAT	AGAGAGACAA	AGTGTCTAT	ATAGATATCA	10000
9901	TGACTACAAAT	GATATGTGCC	TATAGACATG	CACCACAGA	AACAAAAGT	TATCTTTTAT	GGATTTGTA	TCTGTAAAG	CACGTGTCA	AATGACATCT	10100
10001	CATGGCCGGT	ATTGAAGGAT	GGTGTCAAAA	ACTCTGGACA	ATGGAAGCTA	ATCTGAAAGT	TTAGATGAAG	TGAAGGCAGA	TTATCGCTTA	GCAGTAAAAA	10200
10101	TTATTAAACG	GTGAACAAC	ATCAATAGAT	GTAAGTAAAC	CAGTCAAAGT	GAAGGGAAA	CATATAATATC	AAGGATCTTT	CAGTTTATAA	GCAAGGTGAT	10300
10201	TGCTAAAGCA	AATAAGAGAT	GCATACAGAA	ATATAGGCCA	TAACTTTAAA	TAGGACCATG	GATAAACACA	ATATTAGATG	ACATTAAAAAC	TAGTGTGAG	10400
10301	TCAATCTGAA	GGAGTGATGC	ATCCTACCCC	TATAAAAAAG	GTCTTGAGAG	AGTTACTCTG	ATATTAGAA	ACTTCTGGCT	GTATACTTA	TACATGCATG	10500
10401	TCAATAGGGA	GTCTATGTCA	AGAATTAGAA	TTTAGGGGAG	AAAGCATATAT	AAAACATTA	CATCAGTCA	GAGATTTTTT	GAATTTAAAA	GGGAAAAATGA	10600
10501	AATCAAAGCA	ACTTCCTTTG	GCAGGGAAC	AGTTATTCAA	ACAACTAAAT	CAGTAGTCTT	CTATAGATCT	TTCTATAGAA	GGACCCCTGA	TTTTTTAACT	10700
10601	GGTAGTAGAT	CTATGGATGA	ACATACCAAT	GCAATTTTGA	GGAGGAGATC	AAATGAAACG	AAAGTAAAGT	TCTTCAAAGC	CTTACTATCA	ATAGAAAAAA	10800
10701	GAGGCAATCA	GCCATGTAGA	TATTTCTGTTA	AAAATATCAG	CTAACATAAA	TCAGAACGAC	AAGCAAAAGT	AACAAGTGAC	ATCAATAGAA	CAGCAGTTAC	10900
10801	ATGAACGTGC	TACACTGACA	ACACTAATGA	GAGATCCCTCA	AGCTGTTTGA	ACTACAGCAG	AAATGAAAG	GAAGTGGGA	TCAATGCAGA	AAACATAACA	11000
10901	CAGTATCTTA	AGTCTTTCCC	CAAAATCAACT	TTTCAGTGAT	AGTGCTATATC	CAAGCTGAA	AAAAGTTGTA	ACATGATATC	AGGACAAAA	TATTTATACCA	11100
11001	CCTGTTTATC	CTCATGGGCT	GAGAGTATTA	TATGAATCAT	TGCCCTTTCA	GTATCTATGA	TGTTGGAGAA	TCTAGGATTA	TTATCTAGAA	TATTTCTCAGT	11200
11101	ACTTATTACA	GAGAACATCC	GCTATTAAATG	GTGAAGATAT	TGACAGGGCT	GTTCCTAAAT	CTCTAGGACT	TTAAGAGAGA	CATCATGGAA	TAATATGGAA	11300
11201	AGTTGTTGAT	AGTATAGAAA	TTCCAATCAA	ATCTAAATG	AGGCTGATAT	TGCAACTAGT	TCTCATTTGA	AAGGATAAT	TATAGAAAAG	TTACAGACTG	11400
11301	ATAGATTTGAG	TAACATCTCC	TAGCATCACT	ACATGTATGG	ATGTCATATA	AGTACTCAAG	AGAAAAATTT	AGTACCTGTT	TATACAGAC	AAATTTCTCTC	11500
11401	ACAGAACTAC	AGAGGTCBA	AGAGTCCAA	AAAGCCCTTG	GGTAGGGTGC	ATAAAGGAC	ACCAGGCTTG	CGACGATTAC	TCAACAAAGT	CTGTCTTTGGG	11600
11501	AAAACAACAA	AGAGAACAGC	TAGAAGCAAT	TGGAAAAATG	AGATGGGTGT	GAGTGTAAAT	TTCTTACATA	GAATATCTGT	CAGTAGTAGA	CCTATGGAAT	11700
11601	AGTTTATGGCA	TTAGCTACAA	ATGTTTAAAA	CCTTTATATC	CTAGGTTTAT	AGTCTCTATTA	ATCAAGCACT	AAGTGAGAGA	TTTGGAATG	AAGATATTAA	11800
11701	TCCCAGCATC	AGTTCAGCT	TATAGAACAA	CAAAATTACCA	TTTCGACACT						

Fig. 38C

rHMPV-GFP (continued)

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11801 CTGGTCTTC CAAATGCAA TCAGCTGTGG AATTAGCATA ATGAGTGTAG TAGAACAAATT AACAGGTAGA AGCCAAAAC AGTTAGTTT AATACCCCAA 11900
11901 TTAGAAGGAA TAGACATTAT GCCACCACCA GTGTTTCAAG GGAATTCAA TTATAAATTA GTAGATAAGA TAACCTCTGA TCAACATATC TTTAGTCCGG 12000
12001 ACAAATAGA TATGTTAACA CTAGGGAATA TGTCTATGCC CACTATATAA GGTAGAAAA CAGATCAGTT CTTAAATAAG AGAGAAATTT ATTTCCATGG 12100
12101 AACAAATCTT ATTGAGTCTT TATCAGCAGC ATGAGCATCT CAATATTCCCT GGATATTAAAC AGAACAAATGC ATAGAAAAATA ATATTTTCAA GAAAGACTGG 12200
12201 GGTGACGGGT TTATATCAGA TCATGCTTTT ATGGACTTCA AAATATTCCCT ATGTGCTTTT AAACTTAAAC TTTTATGTAG TTGGGATCT CAAGGAAAA 12300
12301 ACATTAAAGA TGAAGATATA GTAGATGAAT TATATGATGT AAAATTCCCTA TCACTAGTAG GTTACAATA GTTTTGGAG AAGTTTCAGC AAGTTATGT TTGAACCCAA 12400
12401 GGTAAAGAAA AGGATAATGT TATATGATGT TGTCAATGCC GAAAGTGAT TGGTTGAGAT GCTACATAGG GTTTTAAAGAC TGGTTTATAG AGCAGTTGAG ATCAGCTGAA 12500
12501 TTGCATGAAA TACCTTGGAT TTTGAACTAT ACAGATATGG CACATGCTCT CACAGATTA CAAGTCAATT AAAATCTATT TGCAACTGAT AGAACAAAGC TTATTTTAA 12600
12601 GAATAACTGT TTTGAACTAT AACCTTAACTC AAGTTATGA TCCACAAACA CAATTAGATT ATCAGAAAGA AGTTAATGTG TGAATAATGCA CTGTTAAACC CAATTTTCATC 12700
12701 CCCAATGGTT AACTTAACTC AAGTTATGA TCCACAAACA CAATTAGATT ACTTCCCAA GATAACATTC GAAAGGCTAA AAAATATAGA CACAAGTTCA 12800
12801 AATTATGCTA AAGGGAAGCT AACCAAGAAAT TACATGATAC TATTGCCATG GCAGCATGTT AATAGATATA ACTTTGTCCT TAGTTCTACT GGATGTAAG 12900
12901 TTAGTCTGAA ACATGTATT GGAAACTTGA TAGAAGTCTG AAAGATGACC GTTTTGTACT TTATTGGAGA AGGAGCAGGA AATTGGATGG CCAGAACAGC 13000
13001 ATGTGAATAT CCTGATATTA AATTTGTATA TAGAAGTCTG AAAGATGACC TTGATCATCA TTATCCTCTG GAATACCAGA GAGTGATAGG TGAATTAAGC 13100
13101 AGAATCATAG ATAGTGGTGA AGGACTTTCA ATGGAAACAA CAGACGCAAC TCAAAAACT CATGGGATTT TGATACACAG GGTAAGCAAA GATGCTTTAT 13200
13201 TAATAACTTT ATGTGATGCA GAATTTAAGG ACAGAGATGA TTTTTTTAAAG ATGGTAAATC TATGGAGAAA ACATGTATTA TCATGCAGAA TTTGCATAC 13300
13301 TTATGGGACG GACCTCTATT TATTCGAAA AGAATGCTAC ATACTCTTAA CACTAGGCCA CCACACAGT TTACCTTGCC ATGGAGAAAT ACAAAATCT AAGATGAAA 13400
13401 GGTAGTAAGC TGTGAGTTT TAATGATTTT TATGCTGCAA AAAAATCTGA CAATAAATCA ATTGAAGCTA ATTGTAATC ACTTTGTCA GGGCTAAGAA TACCTATAA 13500
13501 TAGCAGTGTG TAATGATTTT TAGCAGTTT AGAGAGATTT ATTAACACTA CAAAGCAATC ATTCTCTCTGT AGCAACAGTT GCGGTAGCA AGATCATAGA GTCTAAGTG 13600
13601 TAAGAAGGAA CTAGATAGAC AGAGAGATTT ATTAACACTA CAAAGCAATC TTCTCCAAAG GCGCAATTA AATTATGATTT TTTTGAAGCA TTGGAGACA 13700
13701 TTAACAAACA AAGCAAGTAC AATAATTGAT TGGTTAGAAC ATATTTTAAA AAAAAACTGA TCAAAGTAA AGGATACATG CTGTGAAGTA AAAATGAAA 13800
13801 CTTACCCCTAA TATGATTTAA CTAATAGATA ACTTAGGAA TGCAGAGATT TTAATTGATT ATGCAATAT ATGATAGTTA ATTTAAATTA AAAATGAAA 13900
13901 AATGATGAAG ATGACAAAAT AGATGACAAC TTCATACTAT TCTAAATTA TTAATTGATT ATGCAATAT ATGATAGTTA ATTTAAATTA AAAATGAAA 14000
14001 ATCAAAAGTT AAAATTTAAA ACCTATCATT AAGTTTATTA AAAATAAGAA ATTATAATTG AATGTATACG GTTTTGTGC CGT 14083

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Fig. 38D

HMPV strain 75

1 ACGCCGAAAA AACCGGTATA AATTAAATTC CAAACAAAAC GGGACAAAATA 50
 101 TATATTAAAA GAGTCTCAAT ACACATAAAA AAGATAGTGA GGCACACAAA 60
 201 GATATTCTTT ACCTAAACA TACTGATTAC AAGATATGCTG CAGACATAGG 70
 301 TAAGAAATTC AGCGAGTGA GTTCCAGGTGG TTCTAACCAA CAGACTACTCT 80
 401 ACATGGAGTG GAAAAGAGTT GGGTAGAAGA AATAGACAAA GAGGCAAGAA 90
 501 CAGAGGCTT CAGCACCAGA CACACCAATA ATTTTATTAT GTGTAGGTGC 100
 601 CAGTTAGAG AGCTAACAGA GTGCTAAGTG ATGCGCTCAA AAGTACCCCT 110
 701 GAAAGTGAT TACAGGAGTC TATTCATTGA GTATGGGAAA GCTTTAGGCT 120
 801 CAAGCTTATG GAGCCGGTCA AACATGTCTA AGGTGGGGTG TCAATTGCCAG 130
 901 AGCAAGTTAC AGAGGTTTAT GATTTGGTGA GAGAAATGGG TCCTGAATCT 140
 1001 CAATTGCCCC GATTTTGCTA GTGTTGTCT TGGTAATGCT TCAGTCTAG 150
 1101 CAGATGAAA GTTATGCCAG AAGCTTAAAA GAAAGCAACA AATCAACTT 160
 1201 TAAACATGAG TGATGACAA CAAGATGATT ATGAGTAATT AAAAACTGG 170
 1301 AATGAAGCAG CAAAATAGC AGAAGCTTTC CAGAAATCAC TAAAAAGATC 180
 1401 CAGAAACTCT AGAGCTACCT ACCATCAGCA AACCTGCAAG ATCATCTACA 190
 1501 CACAGAAAA CAAACAACA AAACAACAGA TCCTGTTGAA GAAGAGGAAC 200
 1601 AAAAAATCAA AATCTCCAAC CAATGTAAAA AAGAAAGTTT CCTTCACATC 210
 1701 TCTCAGACAA TGAGGAAGAA GACGCAGAGT CCTCAATCTT AACCTTTGAA 220
 1801 AGAGAAGCTA AGCATGATAT TAGGACTGCT TCCTACACTT AACATGCAA 230
 1901 ATAGAGTAA AACTAATAGC AGAAATAATA AAGAACGCAA AGGAAAAAGC 240
 2001 GCAGTGAAA ACTAACCGAG AAGGCAAAAG AACTTAATAA AATTGTTGAA 250
 2101 TCAGGATAAC AATCAAGGAG AAGATATTTA CCAGTTAATC ATGTAGTTTA 260
 2201 CTATATCAAG CATTCCTTAC ACAGCTGCTG TTCAAAGTTGA TCTGGTAGAA 270
 2301 CAACACACA CCAGCGGTTT TGCTCGATCA GCTAAAGACC TTGACAATAA 280
 2401 TCAGCTCAGG GTGCTGCTAT GTCTGTACTT CCCAAAAAAT TCGAAGTAA 290
 2501 CGGTTTGCGA TGTAAAAACA GTTTAATTGA CAACCATGAA ACCATATGGG 300
 2601 TGATCTAAT GCATGTGTG ACTTCATGGA CCTFAGAGAA AATATACCTG 310
 2701 GCACCTGTTG AAGCTGCAAT AAGCAGTGAG GCGACCAAG CATTAACACA 320
 2801 ATCCAAAAGG TATATTCAAG AAACTAGGAG CTGGAACACA AGTGATAGTA 330
 2901 CTGGAGTCAC CAAGGAACAA GATATGTACT AAAATCCAGA TAAAAATAAC 340
 3001 ATTATAGTTA TATAAAAAA TTTAGAACTA GGAAGGTATT AATAGAAAGC 350
 3101 TAACACCTCA GCACGACTA AAGGAAAGTT ATTTAGAGA ATCATGTAGT 360
 3201 TGCTTTTACA TTAGAAGTTG GTGATGTTGA AAATCTTACA TGTACTGATG 370
 3301 GAACCTCAAA CAGTTTCTGC TGATCAGTTA GCGAGAGAAG AACAAATTGA 380
 3401 CCACAGCAGC AGCAGTCACA GCAGGCAATG CGATAGCCAA AACCTAAGG 390
 3501 GGAGTATCC ACACTAGGAA ATGGAGTGCG AGTCCTAGCC ACCGCAAGTAA 400
 3601 AACAAATGTC ACATTGCTGA TCTGAAGATG GCTGTAGCT TCACTCAATT 410
 3701 TAACACCAGC AATATCATTG GACCTAATGA CTGATGCTGA GCTGGCCAGA 420

Fig. 39A

HMPV strain 75 (continued)

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3801	GAACCTGCA	ATGGTGAGGA	GAAGAAGGATT	TGGAATCTTG	ATAGGGGTCT	ACGGAAGCTC	CGTGATTAC	ATGGTCCAGC	TGCCGATCTT	TGGTGTGATA	3900
3901	GATACACCTT	GTTGGATAAT	CAAGCAGCT	CCCTCTTGTT	CAGAAAAAGA	TGGAACCTAT	GCTTGCTCTC	TAAGAGAGGA	TCAAGGGTGG	TATTGTAAAA	4000
4001	ATGCAGGATC	CACCTGTTTAC	TACCACAATA	AAAAAGACTG	CGAAAAACAAG	GGTGATCATG	TTTTTTTGTG	CACAGCTGCA	GGGATCAATG	TTGCTGAGCA	4100
4101	ATCAAGAGAT	TGCAACATCA	ACATATCTAC	AACCAACTAC	CGATGCAAG	TCAGCAGAGG	AAGACACCTC	ATCAGTAAGG	TTGCACTATC	ACCTCTGGT	4200
4201	GCTTTGGTGG	CTTGCTACAA	AGGGTTAGC	TGTTCAATTG	GCATTAATCG	GGTTGGAATG	ATCAACAAC	TACCTAAGG	CTGCTCATAC	ATAACTAAC	4300
4301	AGGACGCAGA	CACCTGTAACA	ATTGACAACA	CTGTGTATCA	ACTTAAGCAAA	GTTGAGGGTG	ACAGCATGT	AATAAAGGG	AGACAGTTT	CATCAGATTT	4400
4401	CGATCCAATC	AAGTTTCTCG	AGGATCAGTT	CAATGTGCG	CTTGATCAAG	TCCTTTGAAA	CATTGAAAA	AGTCAAGCAC	TAGTGAACCA	GTCAAAACAA	4500
4501	ATTCTGAACA	GTGCAGAAA	AGGAACACT	GGCTTCATTA	TGTGAATAT	TTTGATTTGCT	GTTCTTGGGT	TAAACATGAT	TTCACTGAGC	ATCATATCA	4600
4601	TAATCAAAAA	AACAAGGAAA	CCCACAGGG	CACCTCCAGA	GCTGAATGGT	GTTACCAACG	GCGGTTTTAT	ACCGCATAGT	TAGTTAATTA	AAAAATGGGA	4700
4701	CAAAATCATCA	TGTTCTCGTAA	AGCTCCATGC	AAATATGAAG	TACGGGGCAA	GTGCAACAGG	GGAAGTGAGT	GCAAAATTC	CCACAATTAC	TGGAGTTGGC	4800
4801	CTGATAGGTA	TTTATTGTTA	AGATCAAAAT	ATCTCTGAA	TCAGCTTTTA	AGAAACACTG	ATAAGGCTGA	TGGTTTGTC	ATAATATCAG	GAGCAGGTAG	4900
4901	AGAAGACAGG	ACTCAAGACT	TTGTTCTTGG	TTCTACTAAT	GTGGTTCAAG	GGTACATTGA	TGACAAATCA	GGAATAACAA	AGGCTGCAGC	TTGCTATAGT	5000
5001	CTACATAACA	TAATAAACA	GCTACAAGAA	ATAGAAGTAA	AAATAAATAG	AGATAATAAG	CTTCTTGACA	GCAAAACATGT	GGCACTTCAC	AACTTGATAT	5100
5101	TATCCTATAT	GGAGATGAGC	AAACTCCTG	CATCCCTGAT	TAATAACCTTA	AAGAAACTAC	CAAGAGAAA	ACTGAAAAAA	TTAGCGAAAT	TAATAATTGA	5200
5201	TTTATCAGCA	GGAACTGATA	ATGACTCTTC	ATATGCCTTG	CAAGACAGTG	AAAGCACTAA	TCAAGTGCAG	TAAGCATGTT	CCCAAAATCA	TCACCATAGA	5300
5301	GGCAGATGAT	ATGATATGGA	CACACAAGAA	ATTAAAGAG	ACACTGTCTG	ATGGGATAGC	AAAAATCACAC	ACCAATATTT	ACAGTTGTTA	TTTAGAAAA	5400
5401	ATAGAAATAA	TATATGTTAA	AGTTACTTTA	AGTTAGTAAA	AAATAAATAG	AATGGGATAA	ATGACAAATGA	AAACATTAGA	TGTCATAAAA	AGTGACGAT	5500
5501	CCTCAGAAAC	ATGTAATCAA	CTCAAAAAA	TAATAAAAA	ACACTCAGGT	AAATTGCTTA	TTGCATCAAA	ACCGACATG	GCCTTATTGA	CGTCTTCAC	5600
5601	AGTAACAATT	ACTGTCAACT	ATACAAAAGT	AGAAAAATAT	TTGCAGGCAT	GTCAATTTAA	AAATGATATCA	GACAAAAAGG	ACACAAAGCT	AAATACCACA	5700
5701	TCAACACAA	TCAGACCCAT	TCCTGATCTA	AATGCAATAC	AGTACCTGAA	AAGGCTGATT	CAGAAACACA	CCAACCTCTGT	CACAAAAAGC	AGAGATACCT	5800
5801	TTTGAGAAAT	GACACAGCAT	CAATGACAAA	ATATAAATA	ATATAAGTTC	TATGTTTGTG	GGTCTATGAA	TTCAACCAAT	ACAGCTGTG	AAGAACCAC	5900
5901	AGTTCTATGC	GACAAAAAGT	CAAAAAACAT	CACAGAAAA	CATAGGAAAG	CAGAGTGTCA	CCGTCCATAT	ACAACCCAGT	GGTGGTGCCA	TTATCTTTAA	6000
6001	GAGAAAACTC	AGTTTTCAC	ATTAATAATCA	GACAAATCA	TATCTAGATG	TATTAATATA	ATAGTCTAGT	TACTTAAAA	CTCTAAATAT	TGCTAGACT	6100
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7701	GTAAGTTTTT	TCACATATAA	CCAATGTTA	ACATGGAAAG	ATGTGATGTT						

Fig. 39B

HMPV strain 75 (continued)

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Fig. 39C

HMPV strain 75 (continued)

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Fig. 39D

SEQUENCE LISTING

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 HUMAN SERVICES
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 Biacchesi, Stephane
 Buchholz, Ursula
 Skiadopoulos, Mario H.
 Murphy, Brian R.

<120> RECOMBINANT HUMAN METAPNEUMOVIRUS AND ITS USE

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<150> US 60/451,119
 <151> 2003-02-28

<150> US 60/478,667
 <151> 2003-06-13

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 35 40 45

Asn Thr Asp Arg Ala Asp Gly Leu Ser Ile Ile Ser Gly Ala Gly Arg
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Glu Asp Arg Thr Gln Asp Phe Val Leu Gly Ser Thr Asn Val Val Gln
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Gly Tyr Ile Asp Asp Asn Gln Ser Ile Thr Lys Ala Ala Ala Cys Tyr
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Ser Leu His Asn Ile Ile Lys Gln Leu Gln Glu Val Glu Val Arg Gln
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Ala Arg Asp Ser Lys Leu Ser Asp Ser Lys His Val Ala Leu His Asn
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Leu Ile Leu Ser Tyr Met Glu Met Ser Lys Thr Pro Ala Ser Leu Ile
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Asn Asn Leu Lys Arg Leu Pro Arg Glu Lys Leu Lys Lys Leu Ala Lys
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Leu Gln Asp Ser Glu Ser Ile Asn Gln Val Gln
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Ala Leu Lys Leu Ile Leu Ala Leu Leu Thr Phe Phe Thr Ile Thr Ile
 35 40 45

Thr Ile Asn Tyr Ile Lys Val Glu Asn Asn Leu Gln Ile Cys Gln Ser
 50 55 60

Lys Thr Glu Ser Asp Lys Glu Asp Ser Pro Ser Asn Thr Thr Ser Val
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Thr Thr Lys Thr Thr Leu Asp His Asp Ile Thr Gln Tyr Phe Lys Arg
 85 90 95

Leu Ile Gln Arg Tyr Thr Asp Ser Val Ile Asn Lys Asp Thr Cys Trp
 100 105 110

Lys Ile Ser Arg Asn Gln Cys Thr Asn Ile Thr Thr Tyr Lys Phe Leu

115 120 125
 Cys Phe Lys Pro Glu Asp Ser Lys Ile Asn Ser Cys Asp Arg Leu Thr
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 Asp Leu Cys Arg Asn Lys Ser Lys Ser Ala Ala Glu Ala Tyr His Thr
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 Val Glu Cys His Cys Ile Tyr Thr Ile Glu Trp Lys Cys Tyr His His
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 Ser Ile Asp

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 Leu Ile Leu Ile Gly Ile Thr Thr Leu Ser Ile Ala Leu Asn Ile Tyr
 35 40 45

 Leu Ile Ile Asn Tyr Thr Ile Gln Lys Thr Ser Ser Glu Ser Glu His
 50 55 60

 His Thr Ser Ser Pro Pro Thr Glu Ser Asn Lys Glu Ala Ser Thr Ile
 65 70 75 80

 Ser Thr Asp Asn Pro Asp Ile Asn Pro Asn Ser Gln His Pro Thr Gln
 85 90 95

 Gln Ser Thr Glu Asn Pro Thr Leu Asn Pro Ala Ala Ser Val Ser Pro
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 Ser Glu Thr Glu Pro Ala Ser Thr Pro Asp Thr Thr Asn Arg Leu Ser
 115 120 125

 Ser Val Asp Arg Ser Thr Ala Gln Pro Ser Glu Ser Arg Thr Lys Thr
 130 135 140

Lys Pro Thr Val His Thr Arg Asn Asn Pro Ser Thr Ala Ser Ser Thr
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Gln Ser Pro Pro Arg Ala Thr Thr Lys Ala Ile Arg Arg Ala Thr Thr
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Phe Arg Met Ser Ser Thr Gly Lys Arg Pro Thr Thr Thr Ser Val Gln
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Ser Asp Ser Ser Thr Thr Thr Gln Asn His Glu Glu Thr Gly Ser Ala
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Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Thr Asn Ala Ile Gly Ser
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Cys Leu Leu Lys Arg Pro Tyr Leu Lys Asn Asp Asn Thr Ala Lys Val
 35 40 45

Ala Ile Glu Asn Pro Val Ile Glu His Val Arg Leu Lys Asn Ala Val
 50 55 60

Asn Ser Lys Met Lys Ile Ser Asp Tyr Lys Val Val Glu Pro Val Asn
 65 70 75 80

Met Gln His Glu Ile Met Lys Asn Val His Ser Cys Glu Leu Thr Leu
 85 90 95

Leu Lys Gln Phe Leu Thr Arg Ser Lys Asn Ile Ser Thr Leu Lys Leu
 100 105 110

Asn Met Ile Cys Asp Trp Leu Gln Leu Lys Ser Thr Ser Asp Asp Thr
 115 120 125

Ser Ile Leu Ser Phe Ile Asp Val Glu Phe Ile Pro Ser Trp Val Ser
 130 135 140

Asn Trp Phe Ser Asn Trp Tyr Asn Leu Asn Lys Leu Ile Leu Glu Phe
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Arg Arg Glu Glu Val Ile Arg Thr Gly Ser Ile Leu Cys Arg Ser Leu
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Gly Lys Leu Val Phe Ile Val Ser Ser Tyr Gly Cys Ile Val Lys Ser
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Asn Lys Ser Lys Arg Val Ser Phe Phe Thr Tyr Asn Gln Leu Leu Thr
 195 200 205

Trp Lys Asp Val Met Leu Ser Arg Phe Asn Ala Asn Phe Cys Ile Trp
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Val Ser Asn Ser Leu Asn Glu Asn Gln Glu Gly Leu Gly Leu Arg Ser
 225 230 235 240

Asn Leu Gln Gly Met Leu Thr Asn Lys Leu Tyr Glu Thr Val Asp Tyr
 245 250 255

Met Leu Ser Leu Cys Cys Asn Glu Gly Phe Ser Leu Val Lys Glu Phe
 260 265 270

Glu Gly Phe Ile Met Ser Glu Ile Leu Arg Ile Thr Glu His Ala Gln
 275 280 285

Phe Ser Thr Arg Phe Arg Asn Thr Leu Leu Asn Gly Leu Thr Asp Gln
 290 295 300

Leu Thr Lys Leu Lys Asn Lys Asn Arg Leu Arg Val His Ser Thr Val
 305 310 315 320

Leu Glu Asn Asn Asp Tyr Pro Met Tyr Glu Val Val Leu Lys Leu Leu
 325 330 335

Gly Asp Thr Leu Arg Cys Ile Lys Leu Leu Ile Asn Lys Asn Leu Glu
 340 345 350

Asn Ala Ala Glu Leu Tyr Tyr Ile Phe Arg Ile Phe Gly His Pro Met
 355 360 365

Val Asp Glu Arg Asp Ala Met Asp Ala Val Lys Leu Asn Asn Glu Ile
 370 375 380

Thr Lys Ile Leu Arg Leu Glu Ser Leu Thr Glu Leu Arg Gly Ala Phe
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Ile Leu Arg Ile Ile Lys Gly Phe Val Asp Asn Asn Lys Arg Trp Pro
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Lys Ile Lys Asn Leu Lys Val Leu Ser Lys Arg Trp Thr Met Tyr Phe
 420 425 430

Lys Ala Lys Asn Tyr Pro Ser Gln Leu Glu Leu Ser Glu Gln Asp Phe
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Leu Glu Leu Ala Ala Ile Gln Phe Glu Gln Glu Phe Ser Val Pro Glu
 450 455 460

Lys Thr Asn Leu Glu Met Val Leu Asn Asp Lys Ala Ile Ser Pro Pro
 465 470 475 480

Lys Arg Leu Ile Trp Ser Val Tyr Pro Lys Asn Tyr Leu Pro Glu Thr
 485 490 495

Ile Lys Asn Arg Tyr Leu Glu Glu Thr Phe Asn Ala Ser Asp Ser Leu
 500 505 510

Lys Thr Arg Arg Val Leu Glu Tyr Tyr Leu Lys Asp Asn Lys Phe Asp
 515 520 525

Gln Lys Glu Leu Lys Ser Tyr Val Val Arg Gln Glu Tyr Leu Asn Asp
 530 535 540

Lys Glu His Ile Val Ser Leu Thr Gly Lys Glu Arg Glu Leu Ser Val
 545 550 555 560

Gly Arg Met Phe Ala Met Gln Pro Gly Lys Gln Arg Gln Ile Gln Ile
 565 570 575

Leu Ala Glu Lys Leu Leu Ala Asp Asn Ile Val Pro Phe Phe Pro Glu
 580 585 590

Thr Leu Thr Lys Tyr Gly Asp Leu Asp Leu Gln Arg Ile Met Glu Ile
 595 600 605

Lys Ser Glu Leu Ser Ser Ile Lys Thr Arg Arg Asn Asp Ser Tyr Asn
 610 615 620

Asn Tyr Ile Ala Arg Ala Ser Ile Val Thr Asp Leu Ser Lys Phe Asn
 625 630 635 640

Gln Ala Phe Arg Tyr Glu Thr Thr Ala Ile Cys Ala Asp Val Ala Asp
 645 650 655

Glu Leu His Gly Thr Gln Ser Leu Phe Cys Trp Leu His Leu Ile Val
 660 665 670

Pro Met Thr Thr Met Ile Cys Ala Tyr Arg His Ala Pro Pro Glu Thr
 675 680 685

Lys Gly Glu Tyr Asp Ile Asp Lys Ile Glu Glu Gln Ser Gly Leu Tyr
 690 695 700

Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln Lys Leu Trp Thr
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Met Glu Ala Ile Ser Leu Leu Asp Val Val Ser Val Lys Thr Arg Cys
 725 730 735

Gln Met Thr Ser Leu Leu Asn Gly Asp Asn Gln Ser Ile Asp Val Ser
 740 745 750

Lys Pro Val Lys Leu Ser Glu Gly Leu Asp Glu Val Lys Ala Asp Tyr
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Arg Leu Ala Val Lys Met Leu Lys Glu Ile Arg Asp Ala Tyr Arg Asn
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Ile Gly His Lys Leu Lys Glu Gly Glu Thr Tyr Ile Ser Arg Asp Leu
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Gln Phe Ile Ser Lys Val Ile Gln Ser Glu Gly Val Met His Pro Thr
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Pro Ile Lys Lys Val Leu Arg Val Gly Pro Trp Ile Asn Thr Ile Leu
 820 825 830

Asp Asp Ile Lys Thr Ser Ala Glu Ser Ile Gly Ser Leu Cys Gln Glu
 835 840 845

Leu Glu Phe Arg Gly Glu Ser Ile Ile Val Ser Leu Ile Leu Arg Asn
 850 855 860

Phe Trp Leu Tyr Asn Leu Tyr Met His Glu Ser Lys Gln His Pro Leu
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Ala Gly Lys Gln Leu Phe Lys Gln Leu Asn Lys Thr Leu Thr Ser Val
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Gln Arg Phe Phe Glu Ile Lys Arg Glu Asn Glu Val Val Asp Leu Trp
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Met Asn Ile Pro Met Gln Phe Gly Gly Gly Asp Pro Val Val Phe Tyr
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Arg Ser Phe Tyr Arg Arg Thr Pro Asp Phe Leu Thr Glu Ala Ile Ser
 930 935 940

His Val Asp Ile Leu Leu Lys Ile Ser Ala Asn Ile Lys Asn Glu Thr
 945 950 955 960

Lys Val Ser Phe Phe Lys Ala Leu Leu Ser Ile Glu Lys Asn Glu Arg
 965 970 975

Ala Thr Leu Thr Thr Leu Met Arg Asp Pro Gln Ala Val Gly Ser Glu
 980 985 990

Arg Gln Ala Lys Val Thr Ser Asp Ile Asn Arg Thr Ala Val Thr Ser
 995 1000 1005

Ile Leu Ser Leu Ser Pro Asn Gln Leu Phe Ser Asp Ser Ala Ile
 1010 1015 1020

His Tyr Ser Arg Asn Glu Glu Glu Val Gly Ile Ile Ala Glu Asn
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Ile Thr Pro Val Tyr Pro His Gly Leu Arg Val Leu Tyr Glu Ser
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Leu Pro Phe His Lys Ala Glu Lys Val Val Asn Met Ile Ser Gly
 1055 1060 1065

Thr Lys Ser Ile Thr Asn Leu Leu Gln Arg Thr Ser Ala Ile Asn
 1070 1075 1080

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1100						1105					1110			
Ile	Pro	Ile	Lys	Ser	Asn	Gly	Arg	Leu	Ile	Cys	Cys	Gln	Ile	Ser
1115						1120					1125			
Arg	Thr	Leu	Arg	Glu	Thr	Ser	Trp	Asn	Asn	Met	Glu	Ile	Val	Gly
1130						1135					1140			
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1145						1150					1155			
Thr	Ser	Ser	His	Leu	Lys	Gly	Ile	Ile	Ile	Glu	Lys	Phe	Ser	Thr
1160						1165					1170			
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1175						1180					1185			
Gly	Ser	Ser	Thr	Gln	Glu	Lys	Lys	Leu	Val	Pro	Val	Tyr	Asn	Arg
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Gln	Ile	Leu	Ser	Lys	Gln	Gln	Arg	Glu	Gln	Leu	Glu	Ala	Ile	Gly
1205						1210					1215			
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1235						1240					1245			
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1370						1375					1380			
Met	Leu	Met	Pro	Thr	Ile	Lys	Gly	Gln	Lys	Thr	Asp	Gln	Phe	Leu
1385						1390					1395			
Asn	Lys	Arg	Glu	Asn	Tyr	Phe	His	Gly	Asn	Asn	Leu	Ile	Glu	Ser
1400						1405					1410			
Leu	Ser	Ala	Ala	Leu	Ala	Cys	His	Trp	Cys	Gly	Ile	Leu	Thr	Glu
1415						1420					1425			
Gln	Cys	Ile	Glu	Asn	Asn	Ile	Phe	Lys	Lys	Asp	Trp	Gly	Asp	Gly
1430						1435					1440			
Phe	Ile	Ser	Asp	His	Ala	Phe	Met	Asp	Phe	Lys	Ile	Phe	Leu	Cys
1445						1450					1455			
Val	Phe	Lys	Thr	Lys	Leu	Leu	Cys	Ser	Trp	Gly	Ser	Gln	Gly	Lys
1460						1465					1470			
Asn	Ile	Lys	Asp	Glu	Asp	Ile	Val	Asp	Glu	Ser	Ile	Asp	Lys	Leu
1475						1480					1485			
Leu	Arg	Ile	Asp	Asn	Thr	Phe	Trp	Arg	Met	Phe	Ser	Lys	Val	Met
1490						1495					1500			
Phe	Glu	Pro	Lys	Val	Lys	Lys	Arg	Ile	Met	Leu	Tyr	Asp	Val	Lys
1505						1510					1515			
Phe	Leu	Ser	Leu	Val	Gly	Tyr	Ile	Gly	Phe	Lys	Asn	Trp	Phe	Ile
1520						1525					1530			

Glu	Gln	Leu	Arg	Ser	Ala	Glu	Leu	His	Glu	Ile	Pro	Trp	Ile	Val
1535						1540					1545			
Asn	Ala	Glu	Gly	Asp	Leu	Val	Glu	Ile	Lys	Ser	Ile	Lys	Ile	Tyr
1550						1555					1560			
Leu	Gln	Leu	Ile	Glu	Gln	Ser	Leu	Phe	Leu	Arg	Ile	Thr	Val	Leu
1565						1570					1575			
Asn	Tyr	Thr	Asp	Met	Ala	His	Ala	Leu	Thr	Arg	Leu	Ile	Arg	Lys
1580						1585					1590			
Lys	Leu	Met	Cys	Asp	Asn	Ala	Leu	Leu	Thr	Pro	Ile	Ser	Ser	Pro
1595						1600					1605			
Met	Val	Asn	Leu	Thr	Gln	Val	Ile	Asp	Pro	Thr	Thr	Gln	Leu	Asp
1610						1615					1620			
Tyr	Phe	Pro	Lys	Ile	Thr	Phe	Glu	Arg	Leu	Lys	Asn	Tyr	Asp	Thr
1625						1630					1635			
Ser	Ser	Asn	Tyr	Ala	Lys	Gly	Lys	Leu	Thr	Arg	Asn	Tyr	Met	Ile
1640						1645					1650			
Leu	Leu	Pro	Trp	Gln	His	Val	Asn	Arg	Tyr	Asn	Phe	Val	Phe	Ser
1655						1660					1665			
Ser	Thr	Gly	Cys	Lys	Val	Ser	Leu	Lys	Thr	Cys	Ile	Gly	Lys	Leu
1670						1675					1680			
Met	Lys	Asp	Leu	Asn	Pro	Lys	Val	Leu	Tyr	Phe	Ile	Gly	Glu	Gly
1685						1690					1695			
Ala	Gly	Asn	Trp	Met	Ala	Arg	Thr	Ala	Cys	Glu	Tyr	Pro	Asp	Ile
1700						1705					1710			
Lys	Phe	Val	Tyr	Arg	Ser	Leu	Lys	Asp	Asp	Leu	Asp	His	His	Tyr
1715						1720					1725			
Pro	Leu	Glu	Tyr	Gln	Arg	Val	Ile	Gly	Glu	Leu	Ser	Arg	Ile	Ile
1730						1735					1740			
Asp	Ser	Gly	Glu	Gly	Leu	Ser	Met	Glu	Thr	Thr	Asp	Ala	Thr	Gln
1745						1750					1755			

Lys Thr His Trp Asp Leu Ile His Arg Val Ser Lys Asp Ala Leu	1760	1765	1770
Leu Ile Thr Leu Cys Asp Ala Glu Phe Lys Asp Arg Asp Asp Phe	1775	1780	1785
Phe Lys Met Val Ile Leu Trp Arg Lys His Val Leu Ser Cys Arg	1790	1795	1800
Ile Cys Thr Thr Tyr Gly Thr Asp Leu Tyr Leu Phe Ala Lys Tyr	1805	1810	1815
His Ala Lys Asp Cys Asn Val Lys Leu Pro Phe Phe Val Arg Ser	1820	1825	1830
Val Ala Thr Phe Ile Met Gln Gly Ser Lys Leu Ser Gly Ser Glu	1835	1840	1845
Cys Tyr Ile Leu Leu Thr Leu Gly His His Asn Ser Leu Pro Cys	1850	1855	1860
His Gly Glu Ile Gln Asn Ser Lys Met Lys Ile Ala Val Cys Asn	1865	1870	1875
Asp Phe Tyr Ala Ala Lys Lys Leu Asp Asn Lys Ser Ile Glu Ala	1880	1885	1890
Asn Cys Lys Ser Leu Leu Ser Gly Leu Arg Ile Pro Ile Asn Lys	1895	1900	1905
Lys Glu Leu Asp Arg Gln Arg Arg Leu Leu Thr Leu Gln Ser Asn	1910	1915	1920
His Ser Ser Val Ala Thr Val Gly Gly Ser Lys Ile Ile Glu Ser	1925	1930	1935
Lys Trp Leu Thr Asn Lys Ala Ser Thr Ile Ile Asp Trp Leu Glu	1940	1945	1950
His Ile Leu Asn Ser Pro Lys Gly Glu Leu Asn Tyr Asp Phe Phe	1955	1960	1965
Glu Ala Leu Glu Asn Thr Tyr Pro Asn Met Ile Lys Leu Ile Asp	1970	1975	1980

Asn Leu Gly Asn Ala Glu Ile Lys Lys Leu Ile Lys Val Thr Gly
 1985 1990 1995

Tyr Met Leu Val Ser Lys Lys
 2000 2005

<210> 8
 <211> 177
 <212> PRT
 <213> human metapneumovirus

<400> 8

Met Lys Thr Leu Asp Val Ile Lys Ser Asp Gly Ser Ser Glu Thr Cys
 1 5 10 15

Asn Gln Leu Lys Lys Ile Ile Lys Lys His Ser Gly Lys Leu Leu Ile
 20 25 30

Ala Ser Lys Pro Thr Leu Ala Leu Leu Thr Ser Phe Thr Val Thr Ile
 35 40 45

Thr Val Asn Tyr Thr Lys Val Glu Asn Asn Leu Gln Ala Cys Gln Leu
 50 55 60

Lys Asn Glu Ser Asp Lys Lys Asp Thr Lys Leu Asn Thr Thr Ser Thr
 65 70 75 80

Thr Ile Arg Pro Ile Pro Asp Leu Asn Ala Val Gln Tyr Leu Lys Arg
 85 90 95

Leu Ile Gln Lys His Thr Asn Ser Val Thr Lys Asp Arg Asp Thr Cys
 100 105 110

Trp Arg Ile His Thr Asn Gln Cys Thr Asn Ile Lys Ile Tyr Lys Phe
 115 120 125

Leu Cys Phe Gly Ser Met Asn Ser Thr Asn Thr Asp Cys Glu Glu Pro
 130 135 140

Thr Val Leu Cys Asp Lys Lys Ser Lys Thr Met Thr Glu Lys His Arg
 145 150 155 160

Lys Ala Glu Cys His Arg Pro His Thr Thr Glu Trp Trp Cys His Tyr
 165 170 175

Leu

<210> 9
 <211> 236
 <212> PRT
 <213> human metapneumovirus

<400> 9

Met Glu Ala Arg Val Glu Asn Ile Arg Ala Ile Asp Met Phe Lys Ala
 1 5 10 15

Lys Met Lys Asn Arg Ile Arg Ser Ser Lys Cys His Arg Asn Ala Thr
 20 25 30

Leu Ile Leu Ile Gly Ser Thr Ala Pro Ser Met Ala Leu Asn Thr Leu
 35 40 45

Leu Ile Ile Asp His Ala Thr Ser Lys Asn Met Thr Lys Val Glu His
 50 55 60

Cys Val Asn Met Pro Pro Val Glu Pro Ser Lys Lys Thr Pro Met Thr
 65 70 75 80

Ser Ala Ala Asp Pro Asn Thr Lys Pro Asn Pro Gln Gln Ala Thr Gln
 85 90 95

Leu Thr Thr Glu Asp Ser Thr Ser Leu Ala Ala Thr Leu Glu Asp His
 100 105 110

Leu His Thr Gly Thr Thr Pro Thr Pro Asp Ala Thr Val Ser Gln Gln
 115 120 125

Thr Thr Asp Glu His Thr Thr Leu Leu Arg Ser Thr Asn Arg Gln Thr
 130 135 140

Thr Gln Thr Thr Ala Glu Lys Lys Pro Thr Arg Ala Thr Thr Lys Lys
 145 150 155 160

Glu Thr Thr Thr Arg Thr Thr Ser Thr Ala Ala Thr Gln Thr Leu Asn
 165 170 175

Thr Thr Asn Gln Thr Ser Asn Gly Arg Glu Ala Thr Thr Thr Ser Ala
 180 185 190

Arg Ser Arg Asn Asn Ala Thr Thr Gln Ser Ser Asp Gln Thr Thr Gln

38

Pro Thr Asp Glu Thr Gln Ser
180

<210> 11
<211> 236
<212> PRT
<213> human metapneumovirus

<400> 11

Met Glu Val Lys Val Glu Asn Ile Arg Thr Ile Asp Met Leu Lys Ala
1 5 10 15

Arg Val Lys Asn Arg Val Ala Arg Ser Lys Cys Phe Lys Asn Ala Ser
20 25 30

Leu Val Leu Ile Gly Ile Thr Thr Leu Ser Ile Ala Leu Asn Ile Tyr
35 40 45

Leu Ile Ile Asn Tyr Lys Met Gln Lys Asn Thr Ser Glu Ser Glu His
50 55 60

His Thr Ser Ser Ser Pro Met Glu Ser Ser Arg Glu Thr Pro Thr Val
65 70 75 80

Pro Thr Asp Asn Ser Asp Thr Asn Ser Ser Pro Gln His Pro Thr Gln
85 90 95

Gln Ser Thr Glu Gly Ser Thr Leu Tyr Phe Ala Ala Ser Ala Ser Ser
100 105 110

Pro Glu Thr Glu Pro Thr Ser Thr Pro Asp Thr Thr Asn Arg Pro Pro
115 120 125

Phe Val Asp Thr His Thr Thr Pro Pro Ser Ala Ser Arg Thr Lys Thr
130 135 140

Ser Pro Ala Val His Thr Lys Asn Asn Pro Arg Thr Ser Ser Arg Thr
145 150 155 160

His Ser Pro Pro Arg Ala Thr Thr Arg Thr Ala Arg Arg Thr Thr Thr
165 170 175

Leu Arg Thr Ser Ser Thr Arg Lys Arg Pro Ser Thr Ala Ser Val Gln
180 185 190

Pro Asp Ile Ser Ala Thr Thr His Lys Asn Glu Glu Ala Ser Pro Ala
 195 200 205

Ser Pro Gln Thr Ser Ala Ser Thr Thr Arg Ile Gln Arg Lys Ser Val
 210 215 220

Glu Ala Asn Thr Ser Thr Thr Tyr Asn Gln Thr Ser
 225 230 235

<210> 12
 <211> 2005
 <212> PRT
 <213> human metapneumovirus

<400> 12

Met Asp Pro Leu Asn Glu Ser Thr Val Asn Val Tyr Leu Pro Asp Ser
 1 5 10 15

Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Thr Asn Ala Ile Gly Ser
 20 25 30

Cys Leu Leu Lys Arg Pro Tyr Leu Lys Asn Asp Asn Thr Ala Lys Val
 35 40 45

Ala Ile Glu Asn Pro Val Ile Glu His Val Arg Leu Lys Asn Ala Val
 50 55 60

Asn Ser Lys Met Lys Ile Ser Asp Tyr Lys Ile Val Glu Pro Val Asn
 65 70 75 80

Met Gln His Glu Ile Met Lys Asn Val His Ser Cys Glu Leu Thr Leu
 85 90 95

Leu Lys Gln Phe Leu Thr Arg Ser Lys Asn Ile Ser Thr Leu Lys Leu
 100 105 110

Asn Met Ile Cys Asp Trp Leu Gln Leu Lys Ser Thr Ser Asp Asp Thr
 115 120 125

Ser Ile Leu Ser Phe Ile Asp Val Glu Phe Ile Pro Ser Trp Val Ser
 130 135 140

Asn Trp Phe Ser Asn Trp Tyr Asn Leu Asn Lys Leu Ile Leu Glu Phe
 145 150 155 160

Arg Lys Glu Glu Val Ile Arg Thr Gly Ser Ile Leu Cys Arg Ser Leu
 165 170 175

Gly Lys Leu Val Phe Val Val Ser Ser Tyr Gly Cys Ile Val Lys Ser
 180 185 190

Asn Lys Ser Lys Arg Val Ser Phe Phe Thr Tyr Asn Gln Leu Leu Thr
 195 200 205

Trp Lys Asp Val Met Leu Ser Arg Phe Asn Ala Asn Phe Cys Ile Trp
 210 215 220

Val Ser Asn Ser Leu Asn Glu Asn Gln Glu Gly Leu Gly Leu Arg Ser
 225 230 235 240

Asn Leu Gln Gly Ile Leu Thr Asn Lys Leu Tyr Glu Thr Val Asp Tyr
 245 250 255

Met Leu Ser Leu Cys Cys Asn Glu Gly Phe Ser Leu Val Lys Glu Phe
 260 265 270

Glu Gly Phe Ile Met Ser Glu Ile Leu Arg Ile Thr Glu His Ala Gln
 275 280 285

Phe Ser Thr Arg Phe Arg Asn Thr Leu Leu Asn Gly Leu Thr Asp Gln
 290 295 300

Leu Thr Lys Leu Lys Asn Lys Asn Arg Leu Arg Val His Gly Thr Val
 305 310 315 320

Leu Glu Asn Asn Asp Tyr Pro Met Tyr Glu Val Val Leu Lys Leu Leu
 325 330 335

Gly Asp Thr Leu Arg Cys Ile Lys Leu Leu Ile Asn Lys Asn Leu Glu
 340 345 350

Asn Ala Ala Glu Leu Tyr Tyr Ile Phe Arg Ile Phe Gly His Pro Met
 355 360 365

Val Asp Glu Arg Asp Ala Met Asp Ala Val Lys Leu Asn Asn Glu Ile
 370 375 380

Thr Lys Ile Leu Arg Trp Glu Ser Leu Thr Glu Leu Arg Gly Ala Phe
 385 390 395 400

Ile Leu Arg Ile Ile Lys Gly Phe Val Asp Asn Asn Lys Arg Trp Pro
 405 410 415
 Lys Ile Lys Asn Leu Lys Val Leu Ser Lys Arg Trp Thr Met Tyr Phe
 420 425 430
 Lys Ala Lys Ser Tyr Pro Ser Gln Leu Glu Leu Ser Glu Gln Asp Phe
 435 440 445
 Leu Glu Leu Ala Ala Ile Gln Phe Glu Gln Glu Phe Ser Val Pro Glu
 450 455 460
 Lys Thr Asn Leu Glu Met Val Leu Asn Asp Lys Ala Ile Ser Pro Pro
 465 470 475 480
 Lys Arg Leu Ile Trp Ser Val Tyr Pro Lys Asn Tyr Leu Pro Glu Lys
 485 490 495
 Ile Lys Asn Arg Tyr Leu Glu Glu Thr Phe Asn Ala Ser Asp Ser Leu
 500 505 510
 Lys Thr Arg Arg Val Leu Glu Tyr Tyr Leu Lys Asp Asn Lys Phe Asp
 515 520 525
 Gln Lys Glu Leu Lys Ser Tyr Val Val Lys Gln Glu Tyr Leu Asn Asp
 530 535 540
 Lys Asp His Ile Val Ser Leu Thr Gly Lys Glu Arg Glu Leu Ser Val
 545 550 555 560
 Gly Arg Met Phe Ala Met Gln Pro Gly Lys Gln Arg Gln Ile Gln Ile
 565 570 575
 Leu Ala Glu Lys Leu Leu Ala Asp Asn Ile Val Pro Phe Phe Pro Glu
 580 585 590
 Thr Leu Thr Lys Tyr Gly Asp Leu Asp Leu Gln Arg Ile Met Glu Ile
 595 600 605
 Lys Ser Glu Leu Ser Ser Ile Lys Thr Arg Arg Asn Asp Ser Tyr Asn
 610 615 620
 Asn Tyr Ile Ala Arg Ala Ser Ile Val Thr Asp Leu Ser Lys Phe Asn
 625 630 635 640

Gln Ala Phe Arg Tyr Glu Thr Thr Ala Ile Cys Ala Asp Val Ala Asp
 645 650 655

Glu Leu His Gly Thr Gln Ser Leu Phe Cys Trp Leu His Leu Ile Val
 660 665 670

Pro Met Thr Thr Met Ile Cys Ala Tyr Arg His Ala Pro Pro Glu Thr
 675 680 685

Lys Gly Glu Tyr Asp Ile Asp Lys Ile Glu Glu Gln Ser Gly Leu Tyr
 690 695 700

Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln Lys Leu Trp Thr
 705 710 715 720

Met Glu Ala Ile Ser Leu Leu Asp Val Val Ser Val Lys Thr Arg Cys
 725 730 735

Gln Met Thr Ser Leu Leu Asn Gly Asp Asn Gln Ser Ile Asp Val Ser
 740 745 750

Lys Pro Val Lys Leu Ser Glu Gly Leu Asp Glu Val Lys Ala Asp Tyr
 755 760 765

Ser Leu Ala Val Lys Met Leu Lys Glu Ile Arg Asp Ala Tyr Arg Asn
 770 775 780

Ile Gly His Lys Leu Lys Glu Gly Glu Thr Tyr Ile Ser Arg Asp Leu
 785 790 795 800

Gln Phe Ile Ser Lys Val Ile Gln Ser Glu Gly Val Met His Pro Thr
 805 810 815

Pro Ile Lys Lys Ile Leu Arg Val Gly Pro Trp Ile Asn Thr Ile Leu
 820 825 830

Asp Asp Ile Lys Thr Ser Ala Glu Ser Ile Gly Ser Leu Cys Gln Glu
 835 840 845

Leu Glu Phe Arg Gly Glu Ser Ile Ile Val Ser Leu Ile Leu Arg Asn
 850 855 860

Phe Trp Leu Tyr Asn Leu Tyr Met His Glu Ser Lys Gln His Pro Leu
 865 870 875 880

Ala Gly Lys Gln Leu Phe Lys Gln Leu Asn Lys Thr Leu Thr Ser Val
885 890 895

Gln Arg Phe Phe Glu Ile Lys Lys Glu Asn Glu Val Val Asp Leu Trp
900 905 910

Met Asn Ile Pro Met Gln Phe Gly Gly Gly Asp Pro Val Val Phe Tyr
915 920 925

Arg Ser Phe Tyr Arg Arg Thr Pro Asp Phe Leu Thr Glu Ala Ile Ser
930 935 940

His Val Asp Ile Leu Leu Arg Ile Ser Ala Asn Ile Arg Asn Glu Ala
945 950 955 960

Lys Ile Ser Phe Phe Lys Ala Leu Leu Ser Ile Glu Lys Asn Glu Arg
965 970 975

Ala Thr Leu Thr Thr Leu Met Arg Asp Pro Gln Ala Val Gly Ser Glu
980 985 990

Arg Gln Ala Lys Val Thr Ser Asp Ile Asn Arg Thr Ala Val Thr Ser
995 1000 1005

Ile Leu Ser Leu Ser Pro Asn Gln Leu Phe Ser Asp Ser Ala Ile
1010 1015 1020

His Tyr Ser Arg Asn Glu Glu Glu Val Gly Ile Ile Ala Asp Asn
1025 1030 1035

Ile Thr Pro Val Tyr Pro His Gly Leu Arg Val Leu Tyr Glu Ser
1040 1045 1050

Leu Pro Phe His Lys Ala Glu Lys Val Val Asn Met Ile Ser Gly
1055 1060 1065

Thr Lys Ser Ile Thr Asn Leu Leu Gln Arg Thr Ser Ala Ile Asn
1070 1075 1080

Gly Glu Asp Ile Asp Arg Ala Val Ser Met Met Leu Glu Asn Leu
1085 1090 1095

Gly Leu Leu Ser Arg Ile Leu Ser Val Val Val Asp Ser Ile Glu
1100 1105 1110

Ile	Pro	Thr	Lys	Ser	Asn	Gly	Arg	Leu	Ile	Cys	Cys	Gln	Ile	Ser
1115						1120					1125			
Arg	Thr	Leu	Arg	Glu	Thr	Ser	Trp	Asn	Asn	Met	Glu	Ile	Val	Gly
1130						1135					1140			
Val	Thr	Ser	Pro	Ser	Ile	Thr	Thr	Cys	Met	Asp	Val	Ile	Tyr	Ala
1145						1150					1155			
Thr	Ser	Ser	His	Leu	Lys	Gly	Ile	Ile	Ile	Glu	Lys	Phe	Ser	Thr
1160						1165					1170			
Asp	Arg	Thr	Thr	Arg	Gly	Gln	Arg	Gly	Pro	Lys	Ser	Pro	Trp	Val
1175						1180					1185			
Gly	Ser	Ser	Thr	Gln	Glu	Lys	Lys	Leu	Val	Pro	Val	Tyr	Asn	Arg
1190						1195					1200			
Gln	Ile	Leu	Ser	Lys	Gln	Gln	Arg	Glu	Gln	Leu	Glu	Ala	Ile	Gly
1205						1210					1215			
Lys	Met	Arg	Trp	Val	Tyr	Lys	Gly	Thr	Pro	Gly	Leu	Arg	Arg	Leu
1220						1225					1230			
Leu	Asn	Lys	Ile	Cys	Leu	Gly	Ser	Leu	Gly	Ile	Ser	Tyr	Lys	Cys
1235						1240					1245			
Val	Lys	Pro	Leu	Leu	Pro	Arg	Phe	Met	Ser	Val	Asn	Phe	Leu	His
1250						1255					1260			
Arg	Leu	Ser	Val	Ser	Ser	Arg	Pro	Met	Glu	Phe	Pro	Ala	Ser	Val
1265						1270					1275			
Pro	Ala	Tyr	Arg	Thr	Thr	Asn	Tyr	His	Phe	Asp	Thr	Ser	Pro	Ile
1280						1285					1290			
Asn	Gln	Ala	Leu	Ser	Glu	Arg	Phe	Gly	Asn	Glu	Asp	Ile	Asn	Leu
1295						1300					1305			
Val	Phe	Gln	Asn	Ala	Ile	Ser	Cys	Gly	Ile	Ser	Ile	Met	Ser	Val
1310						1315					1320			
Val	Glu	Gln	Leu	Thr	Gly	Arg	Ser	Pro	Lys	Gln	Leu	Val	Leu	Ile
1325						1330					1335			

Pro	Gln	Leu	Glu	Glu	Ile	Asp	Ile	Met	Pro	Pro	Pro	Val	Phe	Gln
1340						1345					1350			
Gly	Lys	Phe	Asn	Tyr	Lys	Leu	Val	Asp	Lys	Ile	Thr	Ser	Asp	Gln
1355						1360					1365			
His	Ile	Phe	Ser	Pro	Asp	Lys	Ile	Asp	Met	Leu	Thr	Leu	Gly	Lys
1370						1375					1380			
Met	Leu	Met	Pro	Thr	Ile	Lys	Gly	Gln	Lys	Thr	Asp	Gln	Phe	Leu
1385						1390					1395			
Asn	Lys	Arg	Glu	Asn	Tyr	Phe	His	Gly	Asn	Asn	Leu	Ile	Glu	Ser
1400						1405					1410			
Leu	Ser	Ala	Ala	Leu	Ala	Cys	His	Trp	Cys	Gly	Ile	Leu	Thr	Glu
1415						1420					1425			
Gln	Cys	Ile	Glu	Asn	Asn	Ile	Phe	Lys	Lys	Asp	Trp	Gly	Asp	Gly
1430						1435					1440			
Phe	Ile	Ser	Asp	His	Ala	Phe	Met	Asp	Phe	Lys	Ile	Phe	Leu	Cys
1445						1450					1455			
Val	Phe	Lys	Thr	Lys	Leu	Leu	Cys	Ser	Trp	Gly	Ser	Gln	Gly	Lys
1460						1465					1470			
Asn	Ile	Lys	Asp	Glu	Asp	Ile	Val	Asp	Glu	Ser	Ile	Asp	Lys	Leu
1475						1480					1485			
Leu	Arg	Ile	Asp	Asn	Thr	Phe	Trp	Arg	Met	Phe	Ser	Lys	Val	Met
1490						1495					1500			
Phe	Glu	Ser	Lys	Val	Lys	Lys	Arg	Ile	Met	Leu	Tyr	Asp	Val	Lys
1505						1510					1515			
Phe	Leu	Ser	Leu	Val	Gly	Tyr	Ile	Gly	Phe	Lys	Asn	Trp	Phe	Ile
1520						1525					1530			
Glu	Gln	Leu	Arg	Ser	Ala	Glu	Leu	His	Glu	Val	Pro	Trp	Ile	Val
1535						1540					1545			
Asn	Ala	Glu	Gly	Asp	Leu	Val	Glu	Ile	Lys	Ser	Ile	Lys	Ile	Tyr
1550						1555					1560			

Leu	Gln	Leu	Ile	Glu	Gln	Ser	Leu	Phe	Leu	Arg	Ile	Thr	Val	Leu
1565						1570					1575			
Asn	Tyr	Thr	Asp	Met	Ala	His	Ala	Leu	Thr	Arg	Leu	Ile	Arg	Lys
1580						1585					1590			
Lys	Leu	Met	Cys	Asp	Asn	Ala	Leu	Leu	Thr	Pro	Ile	Pro	Ser	Pro
1595						1600					1605			
Met	Val	Asn	Leu	Thr	Gln	Val	Ile	Asp	Pro	Thr	Glu	Gln	Leu	Ala
1610						1615					1620			
Tyr	Phe	Pro	Lys	Ile	Thr	Phe	Glu	Arg	Leu	Lys	Asn	Tyr	Asp	Thr
1625						1630					1635			
Ser	Ser	Asn	Tyr	Ala	Lys	Gly	Lys	Leu	Thr	Arg	Asn	Tyr	Met	Ile
1640						1645					1650			
Leu	Leu	Pro	Trp	Gln	His	Val	Asn	Arg	Tyr	Asn	Phe	Val	Phe	Ser
1655						1660					1665			
Ser	Thr	Gly	Cys	Lys	Val	Ser	Leu	Lys	Thr	Cys	Ile	Gly	Lys	Leu
1670						1675					1680			
Met	Lys	Asp	Leu	Asn	Pro	Lys	Val	Leu	Tyr	Phe	Ile	Gly	Glu	Gly
1685						1690					1695			
Ala	Gly	Asn	Trp	Met	Ala	Arg	Thr	Ala	Cys	Glu	Tyr	Pro	Asp	Ile
1700						1705					1710			
Lys	Phe	Val	Tyr	Arg	Ser	Leu	Lys	Asp	Asp	Leu	Asp	His	His	Tyr
1715						1720					1725			
Pro	Leu	Glu	Tyr	Gln	Arg	Val	Ile	Gly	Glu	Leu	Ser	Arg	Ile	Ile
1730						1735					1740			
Asp	Ser	Gly	Glu	Gly	Leu	Ser	Met	Glu	Thr	Thr	Asp	Ala	Thr	Gln
1745						1750					1755			
Lys	Thr	His	Trp	Asp	Leu	Ile	His	Arg	Val	Ser	Lys	Asp	Ala	Leu
1760						1765					1770			
Leu	Ile	Thr	Leu	Cys	Asp	Ala	Glu	Phe	Lys	Asp	Arg	Asp	Asp	Phe
1775						1780					1785			

Phe	Lys	Met	Val	Ile	Leu	Trp	Arg	Lys	His	Val	Leu	Ser	Cys	Arg
1790						1795					1800			
Ile	Cys	Thr	Thr	Tyr	Gly	Thr	Asp	Leu	Tyr	Leu	Phe	Ala	Lys	Tyr
1805						1810					1815			
His	Ala	Lys	Asp	Cys	Asn	Val	Lys	Leu	Pro	Phe	Phe	Val	Arg	Ser
1820						1825					1830			
Val	Ala	Thr	Phe	Ile	Met	Gln	Gly	Ser	Lys	Leu	Ser	Gly	Ser	Glu
1835						1840					1845			
Cys	Tyr	Ile	Leu	Leu	Thr	Leu	Gly	His	His	Asn	Asn	Leu	Pro	Cys
1850						1855					1860			
His	Gly	Glu	Ile	Gln	Asn	Ser	Lys	Met	Lys	Ile	Ala	Val	Cys	Asn
1865						1870					1875			
Asp	Phe	Tyr	Ala	Ala	Lys	Lys	Leu	Asp	Asn	Lys	Ser	Ile	Glu	Ala
1880						1885					1890			
Asn	Cys	Lys	Ser	Leu	Leu	Ser	Gly	Leu	Arg	Ile	Pro	Ile	Asn	Lys
1895						1900					1905			
Lys	Glu	Leu	Asn	Arg	Gln	Arg	Arg	Leu	Leu	Thr	Leu	Gln	Ser	Asn
1910						1915					1920			
His	Ser	Ser	Val	Ala	Thr	Val	Gly	Gly	Ser	Lys	Val	Ile	Glu	Ser
1925						1930					1935			
Lys	Trp	Leu	Thr	Asn	Lys	Ala	Asn	Thr	Ile	Ile	Asp	Trp	Leu	Glu
1940						1945					1950			
His	Ile	Leu	Asn	Ser	Pro	Lys	Gly	Glu	Leu	Asn	Tyr	Asp	Phe	Phe
1955						1960					1965			
Glu	Ala	Leu	Glu	Asn	Thr	Tyr	Pro	Asn	Met	Ile	Lys	Leu	Ile	Asp
1970						1975					1980			
Asn	Leu	Gly	Asn	Ala	Glu	Ile	Lys	Lys	Leu	Ile	Lys	Val	Thr	Gly
1985						1990					1995			
Tyr	Met	Leu	Val	Ser	Lys	Lys								
2000						2005								

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Ser Tyr Leu Lys Gly Val Ile Ser Phe Ser Glu Cys Asn Ala Leu Gly
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Ser Tyr Ile Phe Asn Gly Pro Tyr Leu Lys Asn Asp Tyr Thr Asn Leu
 35 40 45

Ile Ser Arg Gln Asn Pro Leu Ile Glu His Met Asn Leu Lys Lys Leu
 50 55 60

Asn Ile Thr Gln Ser Leu Ile Ser Lys Tyr His Lys Gly Glu Ile Lys
 65 70 75 80

Leu Glu Glu Pro Thr Tyr Phe Gln Ser Leu Leu Met Thr Tyr Lys Ser
 85 90 95

Met Thr Ser Ser Glu Gln Ile Ala Thr Thr Asn Leu Leu Lys Lys Ile
 100 105 110

Ile Arg Arg Ala Ile Glu Ile Ser Asp Val Lys Val Tyr Ala Ile Leu
 115 120 125

Asn Lys Leu Gly Leu Lys Glu Lys Asp Lys Ile Lys Ser Asn Asn Gly
 130 135 140

Gln Asp Glu Asp Asn Ser Val Ile Thr Thr Ile Ile Lys Asp Asp Ile
 145 150 155 160

Leu Ser Ala Val Lys Asp Asn Gln Ser His Leu Lys Ala Asp Lys Asn
 165 170 175

His Ser Thr Lys Gln Lys Asp Thr Ile Lys Thr Thr Leu Leu Lys Lys
 180 185 190

Leu Met Cys Ser Met Gln His Pro Pro Ser Trp Leu Ile His Trp Phe
 195 200 205

Asn Leu Tyr Thr Lys Leu Asn Asn Ile Leu Thr Gln Tyr Arg Ser Asn

210	215	220
Glu Val Lys Asn His Gly Phe Thr Leu Ile Asp Asn Gln Thr Leu Ser 225 230 235 240		
Gly Phe Gln Phe Ile Leu Asn Gln Tyr Gly Cys Ile Val Tyr His Lys 245 250 255		
Glu Leu Lys Arg Ile Thr Val Thr Thr Tyr Asn Gln Phe Leu Thr Trp 260 265 270		
Lys Asp Ile Ser Leu Ser Arg Leu Asn Val Cys Leu Ile Thr Trp Ile 275 280 285		
Ser Asn Cys Leu Asn Thr Leu Asn Lys Ser Leu Gly Leu Arg Cys Gly 290 295 300		
Phe Asn Asn Val Ile Leu Thr Gln Leu Phe Leu Tyr Gly Asp Cys Ile 305 310 315 320		
Leu Lys Leu Phe His Asn Glu Gly Phe Tyr Ile Ile Lys Glu Val Glu 325 330 335		
Gly Phe Ile Met Ser Leu Ile Leu Asn Ile Thr Glu Glu Asp Gln Phe 340 345 350		
Arg Lys Arg Phe Tyr Asn Ser Met Leu Asn Asn Ile Thr Asp Ala Ala 355 360 365		
Asn Lys Ala Gln Lys Asn Leu Leu Ser Arg Val Cys His Thr Leu Leu 370 375 380		
Asp Lys Thr Val Ser Asp Asn Ile Ile Asn Gly Arg Trp Ile Ile Leu 385 390 395 400		
Leu Ser Lys Phe Leu Lys Leu Ile Lys Leu Ala Gly Asp Asn Asn Leu 405 410 415		
Asn Asn Leu Ser Glu Leu Tyr Phe Leu Phe Arg Ile Phe Gly His Pro 420 425 430		
Met Val Asp Glu Arg Gln Ala Met Asp Ala Val Lys Ile Asn Cys Asn 435 440 445		
Glu Thr Lys Phe Tyr Leu Leu Ser Ser Leu Ser Met Leu Arg Gly Ala		

450 455 460
 Phe Ile Tyr Arg Ile Ile Lys Gly Phe Val Asn Asn Tyr Asn Arg Trp
 465 470 475 480
 Pro Thr Leu Arg Asn Ala Ile Val Leu Pro Leu Arg Trp Leu Thr Tyr
 485 490 495
 Tyr Lys Leu Asn Thr Tyr Pro Ser Leu Leu Glu Leu Thr Glu Arg Asp
 500 505 510
 Leu Ile Val Leu Ser Gly Leu Arg Phe Tyr Arg Glu Phe Arg Leu Pro
 515 520 525
 Lys Lys Val Asp Leu Glu Met Ile Ile Asn Asp Lys Ala Ile Ser Pro
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 Pro Lys Asn Leu Ile Trp Thr Ser Phe Pro Arg Asn Tyr Met Pro Ser
 545 550 555 560
 His Ile Gln Asn Tyr Ile Glu His Glu Lys Leu Lys Phe Ser Glu Ser
 565 570 575
 Asp Lys Ser Arg Arg Val Leu Glu Tyr Tyr Leu Arg Asp Asn Lys Phe
 580 585 590
 Asn Glu Cys Asp Leu Tyr Asn Cys Val Val Asn Gln Ser Tyr Leu Asn
 595 600 605
 Asn Pro Asn His Val Val Ser Leu Thr Gly Lys Glu Arg Glu Leu Ser
 610 615 620
 Val Gly Arg Met Phe Ala Met Gln Pro Gly Met Phe Arg Gln Val Gln
 625 630 635 640
 Ile Leu Ala Glu Lys Met Ile Ala Glu Asn Ile Leu Gln Phe Phe Pro
 645 650 655
 Glu Ser Leu Thr Arg Tyr Gly Asp Leu Glu Leu Gln Lys Ile Leu Glu
 660 665 670
 Leu Lys Ala Gly Ile Ser Asn Lys Ser Asn Arg Tyr Asn Asp Asn Tyr
 675 680 685
 Asn Asn Tyr Ile Ser Lys Cys Ser Ile Ile Thr Asp Leu Ser Lys Phe

690	695	700
Asn Gln Ala Phe Arg Tyr Glu Thr Ser Cys Ile Cys Ser Asp Val Leu 705	710	715 720
Asp Glu Leu His Gly Val Gln Ser Leu Phe Ser Trp Leu His Leu Thr 725	730	735
Ile Pro His Val Thr Ile Ile Cys Thr Tyr Arg His Ala Pro Pro Tyr 740	745	750
Ile Gly Asp His Ile Val Asp Leu Asn Asn Val Asp Glu Gln Ser Gly 755	760	765
Leu Tyr Arg Tyr His Met Gly Gly Ile Glu Gly Trp Cys Gln Lys Leu 770	775	780
Trp Thr Ile Glu Ala Ile Ser Leu Leu Asp Leu Ile Ser Leu Lys Gly 785	790	795 800
Lys Phe Ser Ile Thr Ala Leu Ile Asn Gly Asp Asn Gln Ser Ile Asp 805	810	815
Ile Ser Lys Pro Ile Arg Leu Met Glu Gly Gln Thr His Ala Gln Ala 820	825	830
Asp Tyr Leu Leu Ala Leu Asn Ser Leu Lys Leu Leu Tyr Lys Glu Tyr 835	840	845
Ala Gly Ile Gly His Lys Leu Lys Gly Thr Glu Thr Tyr Ile Ser Arg 850	855	860
Asp Met Gln Phe Met Ser Lys Thr Ile Gln His Asn Gly Val Tyr Tyr 865	870	875 880
Pro Ala Ser Ile Lys Lys Val Leu Arg Val Gly Pro Trp Ile Asn Thr 885	890	895
Ile Leu Asp Asp Phe Lys Val Ser Leu Glu Ser Ile Gly Ser Leu Thr 900	905	910
Gln Glu Leu Glu Tyr Arg Gly Glu Ser Leu Leu Cys Ser Leu Ile Phe 915	920	925
Arg Asn Val Trp Leu Tyr Asn Gln Ile Ala Leu Gln Leu Lys Asn His		

930	935	940
Ala Leu Cys Asn Asn Lys Leu Tyr Leu Asp Ile Leu Lys Val Leu Lys		
945	950	955 960
His Leu Lys Thr Phe Phe Asn Leu Asp Asn Ile Asp Thr Ala Leu Thr		
	965	970 975
Leu Tyr Met Asn Leu Pro Met Leu Phe Gly Gly Gly Asp Pro Asn Leu		
	980	985 990
Leu Tyr Arg Ser Phe Tyr Arg Arg Thr Pro Asp Phe Leu Thr Glu Ala		
	995	1000 1005
Ile Val His Ser Val Phe Ile Leu Ser Tyr Tyr Thr Asn His Asp		
	1010	1015 1020
Leu Lys Asp Lys Leu Gln Asp Leu Ser Asp Asp Arg Leu Asn Lys		
	1025	1030 1035
Phe Leu Thr Cys Ile Ile Thr Phe Asp Lys Asn Pro Asn Ala Glu		
	1040	1045 1050
Phe Val Thr Leu Met Arg Asp Pro Gln Ala Leu Gly Ser Glu Arg		
	1055	1060 1065
Gln Ala Lys Ile Thr Ser Glu Ile Asn Arg Leu Ala Val Thr Glu		
	1070	1075 1080
Val Leu Ser Thr Ala Pro Asn Lys Ile Phe Ser Lys Ser Ala Gln		
	1085	1090 1095
His Tyr Thr Thr Thr Glu Ile Asp Leu Asn Asp Ile Met Gln Asn		
	1100	1105 1110
Ile Glu Pro Thr Tyr Pro His Gly Leu Arg Val Val Tyr Glu Ser		
	1115	1120 1125
Leu Pro Phe Tyr Lys Ala Glu Lys Ile Val Asn Leu Ile Ser Gly		
	1130	1135 1140
Thr Lys Ser Ile Thr Asn Ile Leu Glu Lys Thr Ser Ala Ile Asp		
	1145	1150 1155
Leu Thr Asp Ile Asp Arg Ala Thr Glu Met Met Arg Lys Asn Ile		

1160		1165		1170
Thr Leu Leu Ile Arg Ile	Leu Pro Leu Asp Cys Asn	Arg Asp Lys		
1175	1180	1185		
Arg Glu Ile Leu Ser Met	Glu Asn Leu Ser Ile Thr	Glu Leu Ser		
1190	1195	1200		
Lys Tyr Val Arg Glu Arg	Ser Trp Ser Leu Ser Asn	Ile Val Gly		
1205	1210	1215		
Val Thr Ser Pro Ser Ile	Met Tyr Thr Met Asp Ile	Lys Tyr Thr		
1220	1225	1230		
Thr Ser Thr Ile Ser Ser	Gly Ile Ile Ile Glu Lys	Tyr Asn Val		
1235	1240	1245		
Asn Ser Leu Thr Arg Gly	Glu Arg Gly Pro Thr Lys	Pro Trp Val		
1250	1255	1260		
Gly Ser Ser Thr Gln Glu	Lys Lys Thr Met Pro Val	Tyr Asn Arg		
1265	1270	1275		
Gln Val Leu Thr Lys Lys	Gln Arg Asp Gln Ile Asp	Leu Leu Ala		
1280	1285	1290		
Lys Leu Asp Trp Val Tyr	Ala Ser Ile Asp Asn Lys	Asp Glu Phe		
1295	1300	1305		
Met Glu Glu Leu Ser Ile	Gly Thr Leu Gly Leu Thr	Tyr Glu Lys		
1310	1315	1320		
Ala Lys Lys Leu Phe Pro	Gln Tyr Leu Ser Val Asn	Tyr Leu His		
1325	1330	1335		
Arg Leu Thr Val Ser Ser	Arg Pro Cys Glu Phe Pro	Ala Ser Ile		
1340	1345	1350		
Pro Ala Tyr Arg Thr Thr	Asn Tyr His Phe Asp Thr	Ser Pro Ile		
1355	1360	1365		
Asn Arg Ile Leu Thr Glu	Lys Tyr Gly Asp Glu Asp	Ile Asp Ile		
1370	1375	1380		
Val Phe Gln Asn Cys Ile	Ser Phe Gly Leu Ser Leu	Met Ser Val		

1385		1390		1395
Val Glu Gln Phe Thr Asn	Val Cys Pro Asn Arg Ile	Ile Leu Ile		
1400	1405	1410		
Pro Lys Leu Asn Glu Ile His	Leu Met Lys Pro	Pro Ile Phe Thr		
1415	1420	1425		
Gly Asp Val Asp Ile His	Lys Leu Lys Gln Val Ile	Gln Lys Gln		
1430	1435	1440		
His Met Phe Leu Pro Asp	Lys Ile Ser Leu Thr Gln	Tyr Val Glu		
1445	1450	1455		
Leu Phe Leu Ser Asn Lys	Thr Leu Lys Ser Gly Ser	His Val Asn		
1460	1465	1470		
Ser Asn Leu Ile Leu Ala His	Lys Ile Ser Asp Tyr	Phe His Asn		
1475	1480	1485		
Thr Tyr Ile Leu Ser Thr	Asn Leu Ala Gly His Trp	Ile Leu Ile		
1490	1495	1500		
Ile Gln Leu Met Lys Asp	Ser Lys Gly Ile Phe Glu	Lys Asp Trp		
1505	1510	1515		
Gly Glu Gly Tyr Ile Thr	Asp His Met Phe Ile Asn	Leu Lys Val		
1520	1525	1530		
Phe Phe Asn Ala Tyr Lys	Thr Tyr Leu Leu Cys Phe	His Lys Gly		
1535	1540	1545		
Tyr Gly Lys Ala Lys Leu	Glu Cys Asp Met Asn Thr	Ser Asp Leu		
1550	1555	1560		
Leu Cys Val Leu Glu Leu	Ile Asp Ser Ser Tyr Trp	Lys Ser Met		
1565	1570	1575		
Ser Lys Val Phe Leu Glu	Gln Lys Val Ile Lys Tyr	Ile Leu Ser		
1580	1585	1590		
Gln Asp Ala Ser Leu His	Arg Val Lys Gly Cys His	Ser Phe Lys		
1595	1600	1605		
Leu Trp Phe Leu Lys Arg	Leu Asn Val Ala Glu Phe	Thr Val Cys		

1610		1615		1620
Pro Trp Val Val Asn Ile Asp Tyr His Pro Thr His Met Lys Ala				
1625		1630		1635
Ile Leu Thr Tyr Ile Asp Leu Val Arg Met Gly Leu Ile Asn Ile				
1640		1645		1650
Asp Arg Ile His Ile Lys Asn Lys His Lys Phe Asn Asp Glu Phe				
1655		1660		1665
Tyr Thr Ser Asn Leu Phe Tyr Ile Asn Tyr Asn Phe Ser Asp Asn				
1670		1675		1680
Thr His Leu Leu Thr Lys His Ile Arg Ile Ala Asn Ser Glu Leu				
1685		1690		1695
Glu Asn Asn Tyr Asn Lys Leu Tyr His Pro Thr Pro Glu Thr Leu				
1700		1705		1710
Glu Asn Ile Leu Ala Asn Pro Ile Lys Ser Asn Asp Lys Lys Thr				
1715		1720		1725
Leu Asn Asp Tyr Cys Ile Gly Lys Asn Val Asp Ser Ile Met Leu				
1730		1735		1740
Pro Leu Leu Ser Asn Lys Lys Leu Ile Lys Ser Ser Ala Met Ile				
1745		1750		1755
Arg Thr Asn Tyr Ser Lys Gln Asp Leu Tyr Asn Leu Phe Pro Met				
1760		1765		1770
Val Val Ile Asp Arg Ile Ile Asp His Ser Gly Asn Thr Ala Lys				
1775		1780		1785
Ser Asn Gln Leu Tyr Thr Thr Thr Ser His Gln Ile Ser Leu Val				
1790		1795		1800
His Asn Ser Thr Ser Leu Tyr Cys Met Leu Pro Trp His His Ile				
1805		1810		1815
Asn Arg Phe Asn Phe Val Phe Ser Ser Thr Gly Cys Lys Ile Ser				
1820		1825		1830
Ile Glu Tyr Ile Leu Lys Asp Leu Lys Ile Lys Asp Pro Asn Cys				

1835		1840		1845
Ile Ala Phe Ile Gly Glu Gly Ala Gly Asn Leu Leu Leu Arg Thr				
1850		1855		1860
Val Val Glu Leu His Pro Asp Ile Arg Tyr Ile Tyr Arg Ser Leu				
1865		1870		1875
Lys Asp Cys Asn Asp His Ser Leu Pro Ile Glu Phe Leu Arg Leu				
1880		1885		1890
Tyr Asn Gly His Ile Asn Ile Asp Tyr Gly Glu Asn Leu Thr Ile				
1895		1900		1905
Pro Ala Thr Asp Ala Thr Asn Asn Ile His Trp Ser Tyr Leu His				
1910		1915		1920
Ile Lys Phe Ala Glu Pro Ile Ser Leu Phe Val Cys Asp Ala Glu				
1925		1930		1935
Leu Ser Val Thr Val Asn Trp Ser Lys Ile Ile Ile Glu Trp Ser				
1940		1945		1950
Lys His Val Arg Lys Cys Lys Tyr Cys Ser Ser Val Asn Lys Cys				
1955		1960		1965
Met Leu Ile Val Lys Tyr His Ala Gln Asp Asp Ile Asp Phe Lys				
1970		1975		1980
Leu Asp Asn Ile Thr Ile Leu Lys Thr Tyr Val Cys Leu Gly Ser				
1985		1990		1995
Lys Leu Lys Gly Ser Glu Val Tyr Leu Val Leu Thr Ile Gly Pro				
2000		2005		2010
Ala Asn Ile Phe Pro Val Phe Asn Val Val Gln Asn Ala Lys Leu				
2015		2020		2025
Ile Leu Ser Arg Thr Lys Asn Phe Ile Met Pro Lys Lys Ala Asp				
2030		2035		2040
Lys Glu Ser Ile Asp Ala Asn Ile Lys Ser Leu Ile Pro Phe Leu				
2045		2050		2055
Cys Tyr Pro Ile Thr Lys Lys Gly Ile Asn Thr Ala Leu Ser Lys				

2060	2065	2070
Leu Lys Ser Val Val Ser Gly Asp Ile Leu Ser Tyr Ser Ile Ala 2075 2080 2085		
Gly Arg Asn Glu Val Phe Ser Asn Lys Leu Ile Asn His Lys His 2090 2095 2100		
Met Asn Ile Leu Lys Trp Phe Asn His Val Leu Asn Phe Arg Ser 2105 2110 2115		
Thr Glu Leu Asn Tyr Asn His Leu Tyr Met Val Glu Ser Thr Tyr 2120 2125 2130		
Pro Tyr Leu Ser Glu Leu Leu Asn Ser Leu Thr Thr Asn Glu Leu 2135 2140 2145		
Lys Lys Leu Ile Lys Ile Thr Gly Ser Leu Leu Tyr Asn Phe His 2150 2155 2160		
Asn Glu 2165		
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Glu Cys His Leu Asn Ser Pro Ile Val Lys Gly Lys Ile Ala Gln Leu 20 25 30		
His Thr Ile Met Ser Leu Pro Gln Pro Tyr Asp Met Asp Asp Asp Ser 35 40 45		
Ile Leu Val Ile Thr Arg Gln Lys Ile Lys Leu Asn Lys Leu Asp Lys 50 55 60		
Arg Gln Arg Ser Ile Arg Arg Leu Lys Leu Ile Leu Thr Glu Lys Val 65 70 75 80		
Asn Asp Leu Gly Lys Tyr Thr Phe Ile Arg Tyr Pro Glu Met Ser Lys 85 90 95		
58		

Glu Met Phe Lys Leu His Ile Pro Gly Ile Asn Ser Lys Val Thr Glu
 100 105 110

Leu Leu Leu Lys Ala Asp Arg Thr Tyr Ser Gln Met Thr Asp Gly Leu
 115 120 125

Arg Asp Leu Trp Ile Asn Val Leu Ser Lys Leu Ala Ser Lys Asn Asp
 130 135 140

Gly Ser Asn Tyr Asp Leu Asn Glu Glu Ile Asn Asn Ile Ser Lys Val
 145 150 155 160

His Thr Thr Tyr Lys Ser Asp Lys Trp Tyr Asn Pro Phe Lys Thr Trp
 165 170 175

Phe Thr Ile Lys Tyr Asp Met Arg Arg Leu Gln Lys Ala Arg Asn Glu
 180 185 190

Val Thr Phe Asn Met Gly Lys Asp Tyr Asn Leu Leu Glu Asp Gln Lys
 195 200 205

Asn Phe Leu Leu Ile His Pro Glu Leu Val Leu Ile Leu Asp Lys Gln
 210 215 220

Asn Tyr Asn Gly Tyr Leu Ile Thr Pro Glu Leu Val Leu Pro Tyr Cys
 225 230 235 240

Asp Val Val Glu Gly Arg Trp Asn Ile Ser Ala Cys Ala Lys Leu Asp
 245 250 255

Pro Lys Leu Gln Ser Met Tyr Gln Lys Gly Asn Asn Leu Trp Glu Val
 260 265 270

Ile Asp Lys Leu Phe Pro Ile Met Gly Glu Lys Thr Phe Asp Val Ile
 275 280 285

Ser Leu Leu Glu Pro Leu Ala Leu Ser Leu Ile Gln Thr His Asp Pro
 290 295 300

Val Lys Gln Leu Arg Gly Ala Phe Leu Asn His Val Leu Ser Glu Met
 305 310 315 320

Glu Leu Ile Phe Glu Ser Arg Glu Ser Ile Lys Glu Phe Leu Ser Val
 325 330 335

Asp Tyr Ile Asp Lys Ile Leu Asp Ile Phe Asn Lys Ser Thr Ile Asp
 340 345 350

Glu Ile Ala Glu Ile Phe Ser Phe Phe Arg Thr Phe Gly His Pro Pro
 355 360 365

Leu Glu Ala Ser Ile Ala Ala Glu Lys Val Arg Lys Tyr Met Tyr Ile
 370 375 380

Gly Lys Gln Leu Lys Phe Asp Thr Ile Asn Lys Cys His Ala Ile Phe
 385 390 395 400

Cys Thr Ile Ile Ile Asn Gly Tyr Arg Glu Arg His Gly Gly Gln Trp
 405 410 415

Pro Pro Val Thr Leu Pro Asp His Ala His Glu Phe Ile Ile Asn Ala
 420 425 430

Tyr Gly Ser Asn Ser Ala Ile Ser Tyr Glu Asn Ala Val Asp Tyr Tyr
 435 440 445

Gln Ser Phe Ile Gly Ile Lys Phe Asn Lys Phe Ile Glu Pro Gln Leu
 450 455 460

Asp Glu Asp Leu Thr Ile Tyr Met Lys Asp Lys Ala Leu Ser Pro Lys
 465 470 475 480

Lys Ser Asn Trp Asp Thr Val Ser Pro Ala Ser Asn Leu Leu Tyr Arg
 485 490 495

Thr Asn Ala Ser Asn Glu Ser Arg Arg Leu Val Glu Lys Phe Ile Ala
 500 505 510

Asp Ser Lys Phe Asp Pro Asn Gln Ile Leu Asp Tyr Val Glu Ser Gly
 515 520 525

Asp Trp Leu Asp Asp Pro Glu Phe Asn Ile Ser Tyr Ser Leu Lys Glu
 530 535 540

Lys Glu Ile Lys Gln Glu Gly Arg Leu Phe Ala Lys Met Thr Tyr Lys
 545 550 555 560

Met Arg Ala Thr Gln Val Leu Ser Glu Thr Leu Leu Ala Asn Asn Ile
 565 570 575

Gly Lys Phe Phe Gln Glu Asn Gly Met Val Lys Gly Glu Ile Glu Leu
 580 585 590

Leu Lys Arg Leu Thr Thr Ile Ser Ile Ser Gly Val Pro Arg Tyr Asn
 595 600 605

Glu Val Tyr Asn Asn Ser Lys Ser His Thr Asp Asp Leu Lys Thr Tyr
 610 615 620

Asn Lys Ile Ser Asn Leu Asn Leu Ser Ser Asn Gln Lys Ser Lys Lys
 625 630 635 640

Phe Glu Phe Lys Ser Thr Asp Ile Tyr Asn Asp Gly Tyr Glu Thr Val
 645 650 655

Ser Cys Phe Leu Thr Thr Asp Leu Lys Lys Tyr Cys Leu Asn Trp Arg
 660 665 670

Tyr Glu Ser Thr Ala Leu Phe Gly Glu Thr Cys Asn Gln Ile Phe Gly
 675 680 685

Leu Asn Lys Leu Phe Asn Trp Leu His Pro Arg Leu Glu Gly Ser Thr
 690 695 700

Ile Tyr Val Gly Asp Pro Tyr Cys Pro Pro Ser Asp Lys Glu His Ile
 705 710 715 720

Ser Leu Glu Asp His Pro Asp Ser Gly Phe Tyr Val His Asn Pro Arg
 725 730 735

Gly Gly Ile Glu Gly Phe Cys Gln Lys Leu Trp Thr Leu Ile Ser Ile
 740 745 750

Ser Ala Ile His Leu Ala Ala Val Arg Ile Gly Val Arg Val Thr Ala
 755 760 765

Met Val Gln Gly Asp Asn Gln Ala Ile Ala Val Thr Thr Arg Val Pro
 770 775 780

Asn Asn Tyr Asp Tyr Arg Val Lys Lys Glu Ile Val Tyr Lys Asp Val
 785 790 795 800

Val Arg Phe Phe Asp Ser Leu Arg Glu Val Met Asp Asp Leu Gly His
 805 810 815

Glu Leu Lys Leu Asn Glu Thr Ile Ile Ser Ser Lys Met Phe Ile Tyr
 820 825 830
 Ser Lys Arg Ile Tyr Tyr Asp Gly Arg Ile Leu Pro Gln Ala Leu Lys
 835 840 845
 Ala Leu Ser Arg Cys Val Phe Trp Ser Glu Thr Val Ile Asp Glu Thr
 850 855 860
 Arg Ser Ala Ser Ser Asn Leu Ala Thr Ser Phe Ala Lys Ala Ile Glu
 865 870 875 880
 Asn Gly Tyr Ser Pro Val Leu Gly Tyr Ala Cys Ser Ile Phe Lys Asn
 885 890 895
 Ile Gln Gln Leu Tyr Ile Ala Leu Gly Met Asn Ile Asn Pro Thr Ile
 900 905 910
 Thr Gln Asn Ile Lys Asp Leu Tyr Phe Arg Asn Pro Asn Trp Met Gln
 915 920 925
 Tyr Ala Ser Leu Ile Pro Ala Ser Val Gly Gly Phe Asn Tyr Met Ala
 930 935 940
 Met Ser Arg Cys Phe Val Arg Asn Ile Gly Asp Pro Ser Val Ala Ala
 945 950 955 960
 Leu Ala Asp Ile Lys Arg Phe Ile Lys Ala Asn Leu Leu Asp Arg Ser
 965 970 975
 Val Leu Tyr Arg Ile Met Asn Gln Glu Pro Gly Glu Ser Ser Phe Leu
 980 985 990
 Asp Trp Ala Ser Asp Pro Tyr Ser Cys Asn Leu Pro Gln Ser Gln Asn
 995 1000 1005
 Ile Thr Thr Met Ile Lys Asn Ile Thr Ala Arg Asn Val Leu Gln
 1010 1015 1020
 Asp Ser Pro Asn Pro Leu Leu Ser Gly Leu Phe Thr Asn Thr Met
 1025 1030 1035
 Ile Glu Glu Asp Glu Glu Leu Ala Glu Phe Leu Met Asp Arg Lys
 1040 1045 1050

Val	Ile	Leu	Pro	Arg	Val	Ala	His	Asp	Ile	Leu	Asp	Asn	Ser	Leu
1055						1060					1065			
Thr	Gly	Ile	Arg	Asn	Ala	Ile	Ala	Gly	Met	Leu	Asp	Thr	Thr	Lys
1070						1075					1080			
Ser	Leu	Ile	Arg	Val	Gly	Ile	Asn	Arg	Gly	Gly	Leu	Thr	Tyr	Ser
1085						1090					1095			
Leu	Leu	Arg	Lys	Ile	Ser	Asn	Tyr	Asp	Leu	Val	Gln	Tyr	Glu	Thr
1100						1105					1110			
Leu	Ser	Arg	Thr	Leu	Arg	Leu	Ile	Val	Ser	Asp	Lys	Ile	Arg	Tyr
1115						1120					1125			
Glu	Asp	Met	Cys	Ser	Val	Asp	Leu	Ala	Ile	Ala	Leu	Arg	Gln	Lys
1130						1135					1140			
Met	Trp	Ile	His	Leu	Ser	Gly	Gly	Arg	Met	Ile	Ser	Gly	Leu	Glu
1145						1150					1155			
Thr	Pro	Asp	Pro	Leu	Glu	Leu	Leu	Ser	Gly	Val	Ile	Ile	Thr	Gly
1160						1165					1170			
Ser	Glu	His	Cys	Lys	Ile	Cys	Tyr	Ser	Ser	Asp	Gly	Thr	Asn	Pro
1175						1180					1185			
Tyr	Thr	Trp	Met	Tyr	Leu	Pro	Gly	Asn	Ile	Lys	Ile	Gly	Ser	Ala
1190						1195					1200			
Glu	Thr	Gly	Ile	Ser	Ser	Leu	Arg	Val	Pro	Tyr	Phe	Gly	Ser	Val
1205						1210					1215			
Thr	Asp	Glu	Arg	Ser	Glu	Ala	Gln	Leu	Gly	Tyr	Ile	Lys	Asn	Leu
1220						1225					1230			
Ser	Lys	Pro	Ala	Lys	Ala	Ala	Ile	Arg	Ile	Ala	Met	Ile	Tyr	Thr
1235						1240					1245			
Trp	Ala	Phe	Gly	Asn	Asp	Glu	Ile	Ser	Trp	Met	Glu	Ala	Ser	Gln
1250						1255					1260			
Ile	Ala	Gln	Thr	Arg	Ala	Asn	Phe	Thr	Leu	Asp	Ser	Leu	Lys	Ile
1265						1270					1275			

Leu	Thr	Pro	Val	Ala	Thr	Ser	Thr	Asn	Leu	Ser	His	Arg	Leu	Lys
1280						1285					1290			
Asp	Thr	Ala	Thr	Gln	Met	Lys	Phe	Ser	Ser	Thr	Ser	Leu	Ile	Arg
1295						1300					1305			
Val	Ser	Arg	Phe	Ile	Thr	Met	Ser	Asn	Asp	Asn	Met	Ser	Ile	Lys
1310						1315					1320			
Glu	Ala	Asn	Glu	Thr	Lys	Asp	Thr	Asn	Leu	Ile	Tyr	Gln	Gln	Ile
1325						1330					1335			
Met	Leu	Thr	Gly	Leu	Ser	Val	Phe	Glu	Tyr	Leu	Phe	Arg	Leu	Glu
1340						1345					1350			
Glu	Thr	Thr	Gly	His	Asn	Pro	Ile	Val	Met	His	Leu	His	Ile	Glu
1355						1360					1365			
Asp	Glu	Cys	Cys	Ile	Lys	Glu	Ser	Phe	Asn	Asp	Glu	His	Ile	Asn
1370						1375					1380			
Pro	Glu	Ser	Thr	Leu	Glu	Leu	Ile	Arg	Tyr	Pro	Glu	Ser	Asn	Glu
1385						1390					1395			
Phe	Ile	Tyr	Asp	Lys	Asp	Pro	Leu	Lys	Asp	Val	Asp	Leu	Ser	Lys
1400						1405					1410			
Leu	Met	Val	Ile	Lys	Asp	His	Ser	Tyr	Thr	Ile	Asp	Met	Asn	Tyr
1415						1420					1425			
Trp	Asp	Asp	Thr	Asp	Ile	Ile	His	Ala	Ile	Ser	Ile	Cys	Thr	Ala
1430						1435					1440			
Ile	Thr	Ile	Ala	Asp	Thr	Met	Ser	Gln	Leu	Asp	Arg	Asp	Asn	Leu
1445						1450					1455			
Lys	Glu	Ile	Ile	Val	Ile	Ala	Asn	Asp	Asp	Asp	Ile	Asn	Ser	Leu
1460						1465					1470			
Ile	Thr	Glu	Phe	Leu	Thr	Leu	Asp	Ile	Leu	Val	Phe	Leu	Lys	Thr
1475						1480					1485			
Phe	Gly	Gly	Leu	Leu	Val	Asn	Gln	Phe	Ala	Tyr	Thr	Leu	Tyr	Ser
1490						1495					1500			

Leu	Lys	Thr	Glu	Gly	Arg	Asp	Leu	Ile	Trp	Asp	Tyr	Ile	Met	Arg
1505						1510					1515			
Thr	Leu	Arg	Asp	Thr	Ser	His	Ser	Ile	Leu	Lys	Val	Leu	Ser	Asn
1520						1525					1530			
Ala	Leu	Ser	His	Pro	Lys	Val	Phe	Lys	Arg	Phe	Trp	Asp	Cys	Gly
1535						1540					1545			
Val	Leu	Asn	Pro	Ile	Tyr	Gly	Pro	Asn	Thr	Ala	Ser	Gln	Asp	Gln
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Ile	Lys	Leu	Ala	Leu	Ser	Ile	Cys	Glu	Tyr	Ser	Leu	Asp	Leu	Phe
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Met	Arg	Glu	Trp	Leu	Asn	Gly	Val	Ser	Leu	Glu	Ile	Tyr	Ile	Cys
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Asp	Ser	Asp	Met	Glu	Val	Ala	Asn	Asp	Arg	Lys	Gln	Ala	Phe	Ile
1595						1600					1605			
Ser	Arg	His	Leu	Ser	Phe	Val	Cys	Cys	Leu	Ala	Glu	Ile	Ala	Ser
1610						1615					1620			
Phe	Gly	Pro	Asn	Leu	Leu	Asn	Leu	Thr	Tyr	Leu	Glu	Arg	Leu	Asp
1625						1630					1635			
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1640						1645					1650			
Leu	Lys	Tyr	Val	Gln	Ile	Ser	Gly	Leu	Leu	Ile	Lys	Ser	Phe	Pro
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Ser	Thr	Val	Thr	Tyr	Val	Arg	Lys	Thr	Ala	Ile	Lys	Tyr	Leu	Arg
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Ile	Arg	Gly	Ile	Ser	Pro	Pro	Glu	Val	Ile	Asp	Asp	Trp	Asp	Pro
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Ile	Glu	Asp	Glu	Asn	Met	Leu	Asp	Asn	Ile	Val	Lys	Thr	Ile	Asn
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Asp	Asn	Cys	Asn	Lys	Asp	Asn	Lys	Gly	Asn	Lys	Ile	Asn	Asn	Phe
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Trp	Gly	Leu	Ala	Leu	Lys	Asn	Tyr	Gln	Val	Leu	Lys	Ile	Arg	Ser
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Gly	Gly	Leu	Thr	Leu	Pro	Gln	Gly	Gly	Asn	Tyr	Leu	Ser	His	Gln
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Leu	Arg	Leu	Phe	Gly	Ile	Asn	Ser	Thr	Ser	Cys	Leu	Lys	Ala	Leu
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Glu	Leu	Ser	Gln	Ile	Leu	Met	Lys	Glu	Val	Asn	Lys	Asp	Gln	Asp
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Arg	Leu	Phe	Leu	Gly	Glu	Gly	Ala	Gly	Ala	Met	Leu	Ala	Cys	Tyr
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Ala	Ile	Gly	Lys	Ser	Glu	Glu	Thr	Val	Leu	His	Glu	His	Tyr	Ser
1910						1915					1920			
Val	Ile	Arg	Ile	Thr	Tyr	Leu	Ile	Gly	Asp	Asp	Asp	Val	Val	Leu
1925						1930					1935			
Ile	Ser	Lys	Ile	Ile	Pro	Thr	Ile	Thr	Pro	Asn	Trp	Ser	Arg	Ile
1940						1945					1950			

Leu Tyr 1955 Leu Tyr Lys 1960 Tyr Lys Asp Val Ser 1965 Ile Ile Ser
 Leu Lys 1970 Thr Ser Asn Pro Ala 1975 Ser Thr Glu Leu Tyr 1980 Leu Ile Ser
 Lys Asp 1985 Ala Tyr Cys Thr Ile 1990 Met Glu Pro Ser Glu 1995 Val Val Leu
 Ser Lys 2000 Leu Lys Arg Leu Ser 2005 Leu Leu Glu Glu Asn 2010 Asn Leu Leu
 Lys Trp 2015 Ile Ile Leu Ser Lys 2020 Lys Lys Asn Asn Glu 2025 Trp Leu His
 His Glu 2030 Ile Lys Glu Gly Glu 2035 Arg Asp Tyr Gly Val 2040 Met Arg Pro
 Tyr His 2045 Met Ala Leu Gln Ile 2050 Phe Gly Phe Gln Ile 2055 Asn Leu Asn
 His Leu 2060 Ala Lys Glu Phe Leu 2065 Ser Thr Pro Asp Leu 2070 Thr Asn Ile
 Asn Asn 2075 Ile Ile Gln Ser Phe 2080 Gln Arg Thr Ile Lys 2085 Asp Val Leu
 Phe Glu 2090 Trp Ile Asn Ile Thr 2095 His Asp Gly Lys Arg 2100 His Lys Leu
 Gly Gly 2105 Arg Tyr Asn Ile Phe 2110 Pro Leu Lys Asn Lys 2115 Gly Lys Leu
 Arg Leu 2120 Leu Ser Arg Arg Leu 2125 Val Leu Ser Trp Ile 2130 Ser Leu Ser
 Leu Ser 2135 Thr Arg Leu Leu Thr 2140 Gly Arg Phe Pro Asp 2145 Glu Lys Phe
 Glu His 2150 Arg Ala Gln Thr Gly 2155 Tyr Val Ser Leu Pro 2160 Asp Thr Asp
 Leu Glu 2165 Ser Leu Lys Leu Leu 2170 Ser Lys Asn Thr Ile 2175 Lys Asn Tyr

Arg Glu Cys Ile Gly Ser Ile Ser Tyr Trp Phe Leu Thr Lys Glu
 2180 2185 2190

Val Lys Ile Leu Met Lys Leu Ile Gly Gly Ala Lys Leu Leu Gly
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Ile Pro Arg Gln Tyr Lys Glu Pro Glu Glu Gln Leu Leu Glu Asp
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Tyr Asn Gln His Asp Glu Phe Asp Ile Asp
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Glu Cys His Leu Asn Ser Pro Ile Val Lys Ser Lys Ile Ala Gln Leu
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His Val Leu Leu Asp Ile Asn Gln Pro Tyr Asp Leu Lys Asp Asn Ser
 35 40 45

Ile Ile Asn Ile Thr Lys Tyr Lys Ile Arg Asn Gly Gly Leu Ser Pro
 50 55 60

Arg Gln Ile Lys Ile Arg Ser Leu Gly Lys Ile Leu Lys Gln Glu Ile
 65 70 75 80

Lys Asp Ile Asp Arg Tyr Thr Phe Glu Pro Tyr Pro Ile Phe Ser Leu
 85 90 95

Glu Leu Leu Arg Leu Asp Ile Pro Glu Ile Cys Asp Lys Ile Arg Ser
 100 105 110

Ile Phe Ser Val Ser Asp Arg Leu Ile Arg Glu Leu Ser Ser Gly Phe
 115 120 125

Gln Glu Leu Trp Leu Asn Ile Leu Arg Gln Leu Gly Cys Val Glu Gly
 130 135 140

Lys Glu Gly Phe Asp Ser Leu Lys Asp Val Asp Ile Ile Pro Asp Ile
 145 150 155 160

Thr Asp Lys Tyr Asn Lys Asn Thr Trp Tyr Arg Pro Phe Leu Thr Trp
 165 170 175

Phe Ser Ile Lys Tyr Asp Met Arg Trp Met Gln Lys Asn Lys Ser Gly
 180 185 190

Asn His Leu Asp Val Ser Asn Ser His Asn Phe Leu Asp Cys Lys Ser
 195 200 205

Tyr Ile Leu Ile Ile Tyr Arg Asp Leu Val Ile Ile Ile Asn Lys Leu
 210 215 220

Lys Leu Thr Gly Tyr Val Leu Thr Pro Glu Leu Val Leu Met Tyr Cys
 225 230 235 240

Asp Val Val Glu Gly Arg Trp Asn Met Ser Ser Ala Gly Arg Leu Asp
 245 250 255

Lys Arg Ser Ser Lys Ile Thr Cys Lys Gly Glu Glu Leu Trp Glu Leu
 260 265 270

Ile Asp Ser Leu Phe Pro Asn Leu Gly Glu Asp Val Tyr Asn Ile Ile
 275 280 285

Ser Leu Leu Glu Pro Leu Ser Leu Ala Leu Ile Gln Leu Asp Asp Pro
 290 295 300

Val Thr Asn Leu Lys Gly Ala Phe Met Arg His Val Leu Thr Glu Leu
 305 310 315 320

His Thr Ile Leu Ile Lys Asp Asn Ile Tyr Thr Asp Ser Glu Ala Asp
 325 330 335

Ser Ile Met Glu Ser Leu Ile Lys Ile Phe Arg Glu Thr Ser Ile Asp
 340 345 350

Glu Lys Ala Glu Ile Phe Ser Phe Phe Arg Thr Phe Gly His Pro Ser
 355 360 365

Leu Glu Ala Ile Thr Ala Ala Asp Lys Val Arg Thr His Met Tyr Ser
 370 375 380

Ser Lys Lys Ile Ile Leu Lys Thr Leu Tyr Glu Cys His Ala Ile Phe
 385 390 395 400

Cys Ala Ile Ile Ile Asn Gly Tyr Arg Glu Arg His Gly Gly Gln Trp
 405 410 415

Pro Pro Cys Glu Phe Pro Asn His Val Cys Leu Glu Leu Lys Asn Ala
 420 425 430

Gln Gly Ser Asn Ser Ala Ile Ser Tyr Glu Cys Ala Val Asp Asn Tyr
 435 440 445

Ser Ser Phe Ile Gly Phe Lys Phe Leu Lys Phe Ile Glu Pro Gln Leu
 450 455 460

Asp Glu Asp Leu Thr Ile Tyr Met Lys Asp Lys Ala Leu Ser Pro Arg
 465 470 475 480

Lys Ala Ala Trp Asp Ser Val Tyr Pro Asp Ser Asn Leu Tyr Tyr Lys
 485 490 495

Val Pro Glu Ser Glu Glu Thr Arg Arg Leu Ile Glu Val Phe Ile Asn
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Asp Asn Asn Phe Asn Pro Ala Asp Ile Ile Asn Tyr Val Glu Ser Gly
 515 520 525

Glu Trp Leu Asn Asp Asp Ser Phe Asn Ile Ser Tyr Ser Leu Lys Glu
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Lys Glu Ile Lys Gln Glu Gly Arg Leu Phe Ala Lys Met Thr Tyr Lys
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Met Arg Ala Val Gln Val Leu Ala Glu Thr Leu Leu Ala Lys Gly Val
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Gly Glu Leu Phe Ser Glu Asn Gly Met Val Lys Gly Glu Ile Asp Leu
 580 585 590

Leu Lys Arg Leu Thr Thr Leu Ser Val Ser Gly Val Pro Arg Ser Asn
 595 600 605

Ser Val Tyr Asn Asn Pro Ile Leu His Glu Lys Leu Ile Lys Asn Met
 610 615 620

Asn Lys Cys Asn Ser Asn Gly Tyr Trp Asp Glu Arg Lys Lys Ser Lys
 625 630 635 640

Asn Glu Phe Lys Ala Ala Asp Ser Ser Thr Glu Gly Tyr Glu Thr Leu
 645 650 655

Ser Cys Phe Leu Thr Thr Asp Leu Lys Lys Tyr Cys Leu Asn Trp Arg
 660 665 670

Phe Glu Ser Thr Ala Leu Phe Gly Gln Arg Cys Asn Glu Ile Phe Gly
 675 680 685

Phe Lys Thr Phe Phe Asn Trp Met His Pro Ile Leu Glu Lys Ser Thr
 690 695 700

Ile Tyr Val Gly Asp Pro Tyr Cys Pro Val Pro Asp Arg Met His Lys
 705 710 715 720

Glu Leu Gln Asp His Asp Asp Thr Gly Ile Phe Ile His Asn Pro Arg
 725 730 735

Gly Gly Ile Glu Gly Tyr Cys Gln Lys Leu Trp Thr Leu Ile Ser Ile
 740 745 750

Ser Ala Ile His Leu Ala Ala Val Lys Val Gly Val Arg Val Ser Ala
 755 760 765

Met Val Gln Gly Asp Asn Gln Ala Ile Ala Val Thr Ser Arg Val Pro
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Val Thr Gln Thr Tyr Lys Gln Lys Lys Thr His Val Tyr Glu Glu Ile
 785 790 795 800

Thr Arg Tyr Phe Gly Ala Leu Arg Glu Val Met Phe Asp Ile Gly His
 805 810 815

Glu Leu Lys Leu Asn Glu Thr Ile Ile Ser Ser Lys Met Phe Val Tyr
 820 825 830

Ser Lys Arg Ile Tyr Tyr Asp Gly Lys Ile Leu Pro Gln Cys Leu Lys
 835 840 845

Ala Leu Thr Arg Cys Val Phe Trp Ser Glu Thr Leu Val Asp Glu Asn
 850 855 860

Arg Ser Ala Cys Ser Asn Ile Ala Thr Ser Ile Ala Lys Ala Ile Glu
 865 870 875 880

Asn Gly Tyr Ser Pro Ile Leu Gly Tyr Cys Ile Ala Leu Phe Lys Thr
 885 890 895

Cys Gln Gln Val Cys Ile Ser Leu Gly Met Thr Ile Asn Pro Thr Ile
 900 905 910

Thr Ser Thr Ile Lys Asp Gln Tyr Phe Lys Gly Lys Asn Trp Leu Arg
 915 920 925

Cys Ala Ile Leu Ile Pro Ala Asn Ile Gly Gly Phe Asn Tyr Met Ser
 930 935 940

Thr Ala Arg Cys Phe Val Arg Asn Ile Gly Asp Pro Ala Val Ala Ala
 945 950 955 960

Leu Ala Asp Leu Lys Arg Phe Ile Lys Ala Gly Leu Leu Asp Lys Gln
 965 970 975

Val Leu Tyr Arg Val Met Asn Gln Glu Pro Gly Asp Ser Ser Phe Leu
 980 985 990

Asp Trp Ala Ser Asp Pro Tyr Ser Cys Asn Leu Pro His Ser Gln Ser
 995 1000 1005

Ile Thr Thr Ile Ile Lys Asn Val Thr Ala Arg Ser Val Leu Gln
 1010 1015 1020

Glu Ser Pro Asn Pro Leu Leu Ser Gly Leu Phe Ser Glu Ser Ser
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Ser Glu Glu Asp Leu Asn Leu Ala Ser Phe Leu Met Asp Arg Lys
 1040 1045 1050

Ala Ile Leu Pro Arg Val Ala His Glu Ile Leu Asp Asn Ser Leu
 1055 1060 1065

Thr Gly Val Arg Glu Ala Ile Ala Gly Met Leu Asp Thr Thr Lys
 1070 1075 1080

Ser Leu Val Arg Ala Ser Val Arg Arg Gly Gly Leu Ser Tyr Ser
 1085 1090 1095

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1130						1135					1140			
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1145						1150					1155			
Thr	Pro	Asp	Pro	Leu	Glu	Leu	Leu	Arg	Gly	Ser	Phe	Ile	Glu	Gly
1160						1165					1170			
Ser	Glu	Ile	Cys	Lys	Phe	Cys	Arg	Ser	Glu	Gly	Asn	Asn	Pro	Met
1175						1180					1185			
Tyr	Thr	Trp	Phe	Tyr	Leu	Pro	Asp	Asn	Ile	Asp	Leu	Asp	Thr	Leu
1190						1195					1200			
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1205						1210					1215			
Thr	Asp	Glu	Arg	Ser	Glu	Ala	Gln	Leu	Gly	Tyr	Val	Lys	Asn	Leu
1220						1225					1230			
Ser	Lys	Pro	Ala	Lys	Ala	Ala	Ile	Arg	Ile	Ala	Met	Val	Tyr	Thr
1235						1240					1245			
Trp	Ala	Tyr	Gly	Thr	Asp	Glu	Ile	Ser	Trp	Met	Glu	Ala	Ala	Leu
1250						1255					1260			
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1265						1270					1275			
Leu	Thr	Pro	Val	Ser	Thr	Ser	Thr	Asn	Leu	Ser	His	Arg	Leu	Arg
1280						1285					1290			
Asp	Thr	Ala	Thr	Gln	Met	Lys	Phe	Ser	Ser	Ala	Thr	Leu	Val	Arg
1295						1300					1305			
Ala	Ser	Arg	Phe	Ile	Thr	Ile	Ser	Asn	Asp	Asn	Met	Ala	Leu	Lys
1310						1315					1320			

Glu	Ala	Gly	Glu	Ser	Lys	Asp	Thr	Asn	Leu	Val	Tyr	Gln	Gln	Ile
1325						1330					1335			
Met	Leu	Thr	Gly	Leu	Ser	Leu	Phe	Glu	Phe	Asn	Met	Arg	Tyr	Lys
1340						1345					1350			
Gln	Gly	Ser	Leu	Ser	Lys	Pro	Met	Ile	Leu	His	Leu	His	Leu	Asn
1355						1360					1365			
Asn	Lys	Cys	Cys	Ile	Ile	Glu	Ser	Pro	Gln	Glu	Leu	Asn	Ile	Pro
1370						1375					1380			
Pro	Arg	Ser	Thr	Leu	Asp	Leu	Glu	Ile	Thr	Gln	Glu	Asn	Asn	Lys
1385						1390					1395			
Leu	Ile	Tyr	Asp	Pro	Asp	Pro	Leu	Lys	Asp	Ile	Asp	Leu	Glu	Leu
1400						1405					1410			
Phe	Ser	Lys	Val	Arg	Asp	Val	Val	His	Thr	Ile	Asp	Met	Asn	Tyr
1415						1420					1425			
Trp	Ser	Asp	Asp	Glu	Ile	Ile	Arg	Ala	Thr	Ser	Ile	Cys	Thr	Ala
1430						1435					1440			
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1445						1450					1455			
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1535						1540					1545			

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	1730					1735					1740			
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Leu	Pro	Phe	Asp	Gly	Arg	Tyr	Leu	Ser	His	Gln	Leu	Arg	Leu	Phe
	1760					1765					1770			

Gly	Ile	Asn	Ser	Thr	Ser	Cys	Leu	Lys	Ala	Leu	Glu	Leu	Thr	Tyr
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1805						1810					1815			
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1820						1825					1830			
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1835						1840					1845			
Leu	Val	Gly	Lys	Lys	Leu	Asn	Asn	Val	Thr	Ser	Leu	Cys	Gln	Arg
1850						1855					1860			
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1865						1870					1875			
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1910						1915					1920			
Ala	Tyr	Leu	Val	Gly	Asp	Lys	Asp	Val	Ile	Leu	Val	Ser	Lys	Ile
1925						1930					1935			
Ala	Pro	Arg	Leu	Gly	Thr	Asp	Trp	Thr	Lys	Gln	Leu	Ser	Leu	Tyr
1940						1945					1950			
Leu	Arg	Tyr	Trp	Arg	Asp	Val	Ser	Leu	Ile	Val	Leu	Lys	Thr	Ser
1955						1960					1965			
Asn	Pro	Ala	Ser	Thr	Glu	Met	Tyr	Leu	Ile	Ser	Lys	Asp	Pro	Lys
1970						1975					1980			
Ser	Asp	Ile	Ile	Glu	Asp	Ser	Asn	Thr	Val	Leu	Ala	Asn	Leu	Leu
1985						1990					1995			

Pro	Leu	Ser	Lys	Glu	Asp	Ser	Ile	Lys	Ile	Glu	Lys	Trp	Ile	Leu
2000						2005					2010			
Val	Glu	Lys	Ala	Lys	Val	His	Asp	Trp	Ile	Val	Arg	Glu	Leu	Lys
2015						2020					2025			
Glu	Gly	Ser	Ala	Ser	Ser	Gly	Met	Leu	Arg	Pro	Tyr	His	Gln	Ala
2030						2035					2040			
Leu	Gln	Ile	Phe	Gly	Phe	Glu	Pro	Asn	Leu	Asn	Lys	Leu	Cys	Arg
2045						2050					2055			
Asp	Phe	Leu	Ser	Thr	Leu	Asn	Ile	Val	Asp	Thr	Lys	Asn	Cys	Ile
2060						2065					2070			
Ile	Thr	Phe	Asp	Arg	Val	Leu	Arg	Asp	Thr	Ile	Phe	Glu	Trp	Thr
2075						2080					2085			
Arg	Ile	Lys	Asp	Ala	Asp	Lys	Lys	Leu	Arg	Leu	Thr	Gly	Lys	Tyr
2090						2095					2100			
Asp	Leu	Tyr	Pro	Leu	Arg	Asp	Ser	Gly	Lys	Leu	Lys	Val	Ile	Ser
2105						2110					2115			
Arg	Arg	Leu	Val	Ile	Ser	Trp	Ile	Ala	Leu	Ser	Met	Ser	Thr	Arg
2120						2125					2130			
Leu	Val	Thr	Gly	Ser	Phe	Pro	Asp	Ile	Lys	Phe	Glu	Ser	Arg	Leu
2135						2140					2145			
Gln	Leu	Gly	Ile	Val	Ser	Ile	Ser	Ser	Arg	Glu	Ile	Lys	Asn	Leu
2150						2155					2160			
Arg	Val	Ile	Ser	Lys	Ile	Val	Ile	Asp	Lys	Phe	Glu	Asp	Ile	Ile
2165						2170					2175			
His	Ser	Val	Thr	Tyr	Arg	Phe	Leu	Thr	Lys	Glu	Ile	Lys	Ile	Leu
2180						2185					2190			
Met	Lys	Ile	Leu	Gly	Ala	Val	Lys	Leu	Phe	Gly	Ala	Arg	Gln	Ser
2195						2200					2205			
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Ser Asp Leu Gly Lys Tyr Thr Phe Ile Arg Tyr Pro Glu Met Ser Ser
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Glu Met Phe Gln Leu Cys Ile Pro Gly Ile Asn Asn Lys Ile Asn Glu
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Leu Leu Ser Lys Ala Ser Lys Thr Tyr Asn Gln Met Thr Asp Gly Leu
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Gly Ser Asn Tyr Asp Ile Asn Glu Asp Ile Ser Asn Ile Ser Asn Val
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His Met Thr Tyr Gln Ser Asp Lys Trp Tyr Asn Pro Phe Lys Thr Trp
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Ile Thr Phe Asn Arg His Lys Asp Tyr Asn Leu Leu Glu Asp Gln Lys
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 Asn Tyr Asn Gly Tyr Ile Met Thr Pro Glu Leu Val Leu Met Tyr Cys
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 Val Lys Gln Leu Arg Gly Ala Phe Leu Asn His Val Leu Ser Glu Met
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 Cys Thr Ile Ile Ile Asn Gly Tyr Arg Glu Arg His Gly Gly Gln Trp
 405 410 415
 Pro Pro Val Thr Leu Pro Val His Ala His Glu Phe Ile Ile Asn Ala
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 Tyr Gly Ser Asn Ser Ala Ile Ser Tyr Glu Asn Ala Val Asp Tyr Tyr
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Lys Ser Phe Ile Gly Ile Lys Phe Asp Lys Phe Ile Glu Pro Gln Leu
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Asp Glu Asp Leu Thr Ile Tyr Met Lys Asp Lys Ala Leu Ser Pro Lys
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Asp Ser Lys Phe Asp Pro His Gln Val Leu Asp Tyr Val Glu Ser Gly
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Tyr Trp Leu Asp Asp Pro Glu Phe Asn Ile Ser Tyr Ser Leu Lys Glu
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Lys Glu Ile Lys Gln Glu Gly Arg Leu Phe Ala Lys Met Thr Tyr Lys
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Met Arg Ala Thr Gln Val Leu Ser Glu Thr Leu Leu Ala Asn Asn Ile
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Gly Lys Phe Phe Gln Glu Asn Gly Met Val Lys Gly Glu Ile Glu Leu
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Leu Lys Arg Leu Thr Thr Ile Ser Met Ser Gly Val Pro Arg Tyr Asn
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Ile Tyr Val Gly Asp Pro Tyr Cys Pro Pro Ser Asp Ile Glu His Leu
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Pro Leu Asp Asp His Pro Asp Ser Gly Phe Tyr Val His Asn Pro Lys
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Gly Gly Ile Glu Gly Phe Cys Gln Lys Leu Trp Thr Leu Ile Ser Ile
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Met Ser Arg Cys Phe Val Arg Asn Ile Gly Asp Pro Thr Val Ala Ala
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Leu Ala Asp Ile Lys Arg Phe Ile Lys Ala Asn Leu Leu Asp Arg Gly
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Met Trp Met His Leu Ser Gly Gly Arg Met Ile Asn Gly Leu Glu
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Tyr	Thr	Trp	Met	Tyr	Leu	Pro	Gly	Asn	Leu	Asn	Ile	Gly	Ser	Ala
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Glu	Thr	Gly	Ile	Ala	Ser	Leu	Arg	Val	Pro	Tyr	Phe	Gly	Ser	Val
	1205					1210					1215			
Thr	Asp	Glu	Arg	Ser	Glu	Ala	Gln	Leu	Gly	Tyr	Ile	Lys	Asn	Leu
	1220					1225					1230			
Ser	Lys	Pro	Ala	Lys	Ala	Ala	Ile	Arg	Ile	Ala	Met	Ile	Tyr	Thr
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Trp	Ala	Phe	Gly	Asn	Asp	Glu	Ile	Ser	Trp	Met	Glu	Ala	Ser	Gln
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Ile	Ala	Gln	Thr	Arg	Ala	Asn	Phe	Thr	Leu	Asp	Ser	Leu	Lys	Ile
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Leu	Thr	Pro	Val	Thr	Thr	Ser	Thr	Asn	Leu	Ser	His	Arg	Leu	Lys
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Asp	Thr	Ala	Thr	Gln	Met	Lys	Phe	Ser	Ser	Thr	Ser	Leu	Ile	Arg
	1295					1300					1305			
Val	Ser	Arg	Phe	Ile	Thr	Ile	Ser	Asn	Asp	Asn	Met	Ser	Ile	Lys
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 1820 1825 1830

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Ile	Leu	Asn	Arg	Val	Arg	Val	Leu	Phe	Asn	Gly	Asn	Pro	Asn	Ser
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Thr	Trp	Ile	Gly	Asn	Met	Glu	Cys	Glu	Ser	Leu	Ile	Trp	Ser	Glu
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1895						1900					1905			
Ala	Ile	Gly	Lys	Ser	Glu	Glu	Thr	Val	Leu	His	Glu	His	Tyr	Ser
1910						1915					1920			
Ile	Ile	Arg	Ile	Thr	Tyr	Leu	Ile	Gly	Asp	Asp	Asp	Val	Val	Leu
1925						1930					1935			
Val	Ser	Lys	Ile	Ile	Pro	Thr	Ile	Thr	Pro	Asn	Trp	Ser	Lys	Ile
1940						1945					1950			
Leu	Tyr	Leu	Tyr	Lys	Leu	Tyr	Trp	Lys	Asp	Val	Ser	Val	Val	Ser
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Leu	Lys	Thr	Ser	Asn	Pro	Ala	Ser	Thr	Glu	Leu	Tyr	Leu	Ile	Ser
1970						1975					1980			
Lys	Asp	Ala	Tyr	Cys	Thr	Val	Met	Glu	Pro	Ser	Asn	Leu	Val	Leu
1985						1990					1995			
Ser	Lys	Leu	Lys	Arg	Ile	Ser	Ser	Ile	Glu	Glu	Asn	Asn	Leu	Leu
2000						2005					2010			
Lys	Trp	Ile	Ile	Leu	Ser	Lys	Arg	Lys	Asn	Asn	Glu	Trp	Leu	Gln
2015						2020					2025			
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His Leu Ala Arg Glu Phe Leu Ser Thr Pro Asp Leu Thr Asn Ile
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Asn Asn Ile Ile Gln Ser Phe Thr Arg Thr Ile Lys Asp Val Met
 2075 2080 2085

Phe Glu Trp Val Asn Ile Thr His Asp Asn Lys Arg His Lys Leu
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Gly Gly Arg Tyr Asn Leu Phe Pro Leu Lys Asn Lys Gly Lys Leu
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Lys Glu His Ile Gly Leu Ile Ser Tyr Trp Phe Leu Thr Lys Glu
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Val Lys Ile Leu Met Lys Leu Ile Gly Gly Val Lys Leu Leu Gly
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